Lung (2009) 187:307–313
DOI 10.1007/s00408-009-9160-8

CYSTIC FIBROSIS

Extended Nitric Oxide Measurements in Exhaled Air of Cystic Fibrosis and Healthy Adults

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Received: 4 April 2009 / Accepted: 5 July 2009 / Published online: 8 August 2009

Abstract In cystic fibrosis (CF) lung disease, exhaled nitric oxide (FeNO) is not raised, but rather is normal or even decreased when measured at a single expiratory flow. FeNO measurements at several flow rates allow differentiation between alveolar and bronchial nitric oxide (NO) production. Extended FeNO measurements therefore should be useful to localize the FeNO deficit in CF airways. FeNO was measured in stable CF adults with moderate lung disease and in healthy controls. Bronchial NO fluxes ($J_{NO,Br}$) and alveolar NO concentrations (C_{Alv}) were calculated from FeNO measurements at flow rates of 100, 150 and 200 ml/s using a method previously described. Thirty-two adults were included in the study, 12 of whom had CF. CF adults had significantly lower FeNO values at all flow rates. The median $J_{NO,Br}$ was significantly lower in CF adults than in healthy controls [0.31 nl/s (range = 0.11–0.63) vs. 0.70 nl/s (0.27–3.52); $P < 0.001$], while the median C_{Alv} was similar in both groups [1.7 ppb (0.3–3.9) vs. 1.2 (0.1–5.2)]. Pulmonary NO exchange did not differ significantly between subgroups of CF patients with and without chronic *Pseudomonas aeruginosa* infection. No significant correlation was detectable between FEV_1/VC and $J_{NO,Br}$ and C_{Alv}, respectively. Extended FeNO measurements can separate alveolar and bronchial NO outputs in CF adults. The lower FeNO in adults with moderate to severe CF lung disease is likely to be the result of lower bronchial NO output.

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Keywords Cystic fibrosis · Adults · Inflammation · Nitric oxide

Introduction

Nitric oxide (NO), an important signalling mediator in many organ systems, is synthesized from L-arginine by nitric oxide synthetase (NOS), which exists in two constitutive (cNOS) and one inducible (iNOS) isoform. The constitutive isoforms are localized in airway nerves and in bronchial epithelium, in type II alveolar epithelial cells, and in endothelial cells of pulmonary blood vessels, where they synthesize low amounts of NO for regulatory purposes [1]. On the other hand, iNOS releases large quantities of NO on induction by proinflammatory cytokines. Inducible NOS immunoreactivity has been shown in many cell types such as neutrophils, eosinophils, macrophages, type II alveolar epithelial cells, airway epithelial cells, mast cells, fibroblasts, endothelial cells, and airway and vascular smooth muscle cells [2]. Thus, it is not surprising that NO serves diverse physiological and cellular functions in the lungs. The measurement of fractional NO concentrations in exhaled air (FeNO) has been utilized to monitor various conditions of the respiratory tract in health and disease [2–4]. In inflammatory lung disease such as asthma, FeNO has been found to be a useful noninvasive surrogate marker of airway inflammation [5, 6].

In cystic fibrosis (CF) airways disease, characterized by neutrophilic airway inflammation and chronic airway infection, nasal and lower-airway FeNO is not raised, but rather is normal or even decreased when measured at a single expiratory flow [7–10]. Unfortunately, the use of constant flow exercises does not allow differentiation if the endogenous NO production originates in the proximal or peripheral

airways of the lungs. A two-compartment model was developed in the 1990s to interpret FeNO measurements with regard to the regional distribution of endogenous NO production [11–14]. According to this paradigm, FeNO is the sum of two contributions, namely, the net NO output of the airways, which are modeled as rigid tubes, and of the alveolar compartment, which is modeled as a flexible balloon [15–17]. The clinical applications of the two-compartment model are quite new. Based on the method of Tsoukias and George [11], Suri et al. [18] have recently found that alveolar, but not bronchial, NO production is elevated in a cohort of CF children with mild lung disease.

To our knowledge, the pulmonary NO exchange dynamics in CF adult patients have not been studied in detail so far using the two-compartment model. Our study aimed to analyze the flow-independent parameters of pulmonary NO exchange in CF adults compared to healthy controls by exploring the origin of the low FeNO previously observed in CF airways.

Patients and Methods

Study Patients and Control Subjects

Adult CF patients were recruited from the Adult CF Unit of the University Hospital Zurich, Switzerland. The diagnosis of CF was based on standard criteria, including each patient's CF genotype. Patients were evaluated only if they were clinically stable and without clinical signs of acute infection exacerbation and had stable lung function at the time of measurement. Chronic *Pseudomonas aeruginosa* infection was defined according to a recently published consensus definition, i.e., at least three positive sputum or cough swab cultures over 6 months or more with a minimum 1-month interval in between [19]. All CF patients with chronic *Pseudomonas aeruginosa* infection received maintenance treatment with oral azithromycin (500 mg three times weekly) and long-term inhalation therapy with colistin (1 million units twice daily) or TOBI® (300 mg twice daily). A subgroup of patients also were treated long-term with nebulized dornase α daily. No CF patient received oral or nebulized steroid treatment for at least 1 month prior to the time of assessment. None of the CF patients had a positive medical history of atopy. Skin prick tests were not performed.

Healthy control subjects recruited for the purpose of the study had no medical history of chronic illnesses, lung disease, and/or atopy and no respiratory tract infection at the time of their lung function measurements. No skin prick tests were performed in control subjects. In addition, control subjects had lung function test results [spirometry, body plethysmograph, carbon monoxide (CO) diffusion capacity] within normal limits. Smokers were excluded from the study.

The Ethics Committee of the University Hospital Zurich approved the study. Written informed consent was obtained from all CF patients and healthy control subjects.

Measurement of Exhaled Nitric Oxide and Pulmonary Function Test

FeNO was measured according to the guidelines of the American Thoracic Society (ATS) and European Respiratory Society (ERS) with a CLD 88-NO analyzer (Eco Medics™, Dürnten, Switzerland), using the chemiluminescent NO measuring method [3]. The analyzer was calibrated daily and readjusted before each measurement. Each subject inhaled NO-free air to total lung capacity and exhaled as long as possible at four different exhalation flow rates (V_E = 50, 100, 150, and 200 ml/s) against a resistance of 10 cm H_2O L^{-1} s^{-1}. The breathing maneuvers were repeated three times at each exhalation flow rate. The different exhalation flow rates were obtained by using four flow restrictors designed to produce a laminar flow of the desired exhalation flow rate at a constant resistance of 10 cm H_2O L^{-1} s^{-1} for velum closure and normal breathing pressure. The exhalation flow rate was measured with an ultrasound flowmeter (Eco Medics), and biofeedback on a computer screen was used to maintain the desired flow. The exhaled NO concentration (FeNO) was registered as a function of exhaled volume (Fig. 1a), while the corresponding NO elimination rate V_{NO}, the product of the measured exhalation flow rate (V_E) and the exhaled NO concentration (FeNO), was recorded as a function of time (Fig. 1b). The dead space of the flowmeter was 20 ml. The end-expiratory FeNO (75–95% of the exhalation cycle) or the corresponding V_{NO}, respectively, was utilized for calculations. Spirometry [forced expiratory volume in 1 s (FEV_1); forced vital capacity (FVC)] was performed on the same day before NO measurements. Pulmonary function tests were performed according to our laboratory protocols, which are based upon adult European Respiratory Society (ERS) standard testing [20, 21].

Calculation of Alveolar NO Concentration (C_{Alv}) and Bronchial NO flux ($J_{NO,Br}$)

The alveolar NO concentration (C_{Alv}) and the bronchial NO flux ($J_{NO,Br}$) were calculated according to the model of Tsoukias and George [11] and as described in detail by Lehtimaki et al. [14, 15]. In the two-compartment model the end-expiratory FeNO at $V_E \geq 100$ ml/s originates mainly from the lower respiratory tract. Therefore, in the end-expiratory phase and at $V_E \geq 100$ ml/s, the NO elimination rate V_{NO} is a linear function of the exhalation

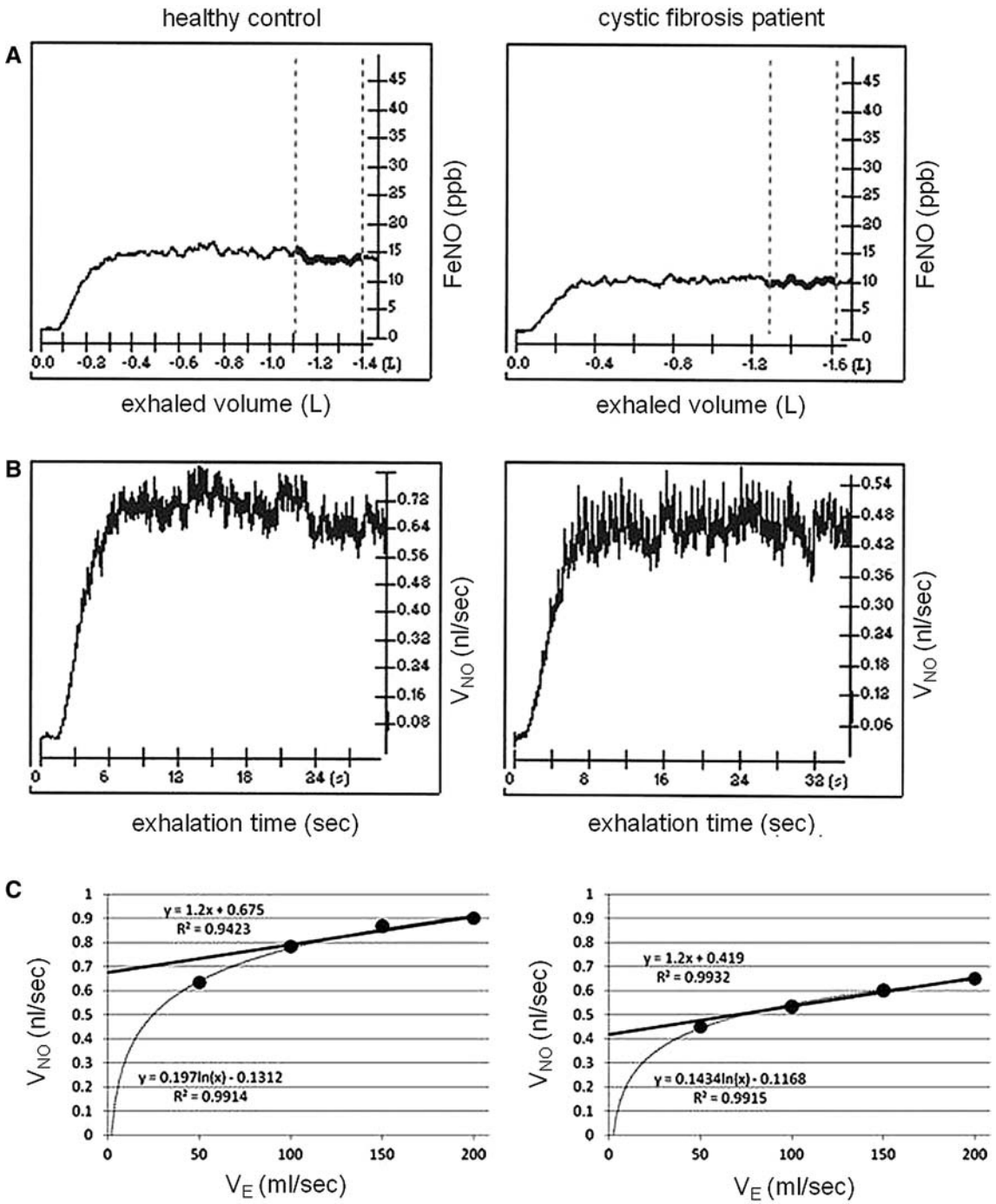

Fig. 1 Measurement of end-expiratory FeNO and calculation of flow-independent parameters. Examples of online measurements at $V_E = 50$ ml/s in rows A (FeNO vs. volume) and B (V_{NO} vs. time) for a healthy control and a CF patient show that a plateau phase is established which allows extraction of end-expiratory FeNO according to the ATS and ERS guidelines. In row C the linear relationship between V_{NO} and V_E at $V_E \geq 100$ ml/s is demonstrated and allows determination of the flow-independent parameters C_{Alv} (slope) and $J_{NO,Br}$ (intercept) according to Refs. [11] and [14]

flow rate V_E, with the slope representing the alveolar NO concentration (C_{Alv}) and a nonzero intercept approximating the bronchial NO flux ($J_{NO,Br}$). In Fig. 1c we show regression lines calculated from data of a healthy control and a CF patient. Although we also measured FeNO at $V_E = 50$ ml/s, as recommended by the ATS and ERS guidelines, only V_{NO} values obtained at $V_E \geq 100$ ml/s were used for the calculation.

Statistics

Values are expressed as mean and 95% CI (confidence interval) of the mean, and, where appropriate, the median and range are presented for skewed data. The Mann–Whitney test was used for statistical calculation. $P < 0.05$ was considered statistically significant. Correlations between bronchial obstruction (FEV_1/VC) and C_{Alv} and $J_{NO,Br}$ were made using Spearman's rank test.

Results

Twelve adult CF patients and 20 healthy control subjects were included in the study. Patient demographics are given in Table 1. Age and gender distribution were similar in the two groups. All except of one patient were ΔF508 homozygous. Patients had moderate to severe CF lung disease (median $FEV_1 = 1.6$ L or 48% predicted), and two-thirds were chronically infected with *Pseudomonas aeruginosa*. At the time of recruitment, none of the CF patients was listed for lung transplantation, even though the patients' mean age was greater than 30 years, which is higher than the mean age of 26.2 years for CF patients transplanted at our center in the last decade [22].

As described above, the end-expiratory FeNO (75-95% of the exhalation cycle) at an exhalation flow rate of $V_E \geq 100$ ml/s was utilized for calculations. The mean standard deviations of end-expiratory FeNO were 0.7 ± 0.4, 0.4 ± 0.3, 0.3 ± 0.2, and 0.3 ± 0.1 ppb for the flow rates of 50, 100, 150, and 200 ml/s, respectively. In CF patients, end-expiratory FeNO was significantly lower compared to that of healthy control subjects at all exhalation flow rates (Fig. 2). There was a negative linear correlation between end-expiratory FeNO and exhalation flow rates $V_E \geq 100$ ml/s observed in both the CF patient group and the control groups (Figs. 1 and 2). Thus, the analytical tools of the two-compartment model can be applied to our data.

Significantly lower bronchial NO fluxes ($J_{NO,Br}$) were documented in CF adults compared to healthy controls, while alveolar NO concentrations (C_{Alv}) were similar in both groups, as displayed in Fig. 3. Similarly, C_{Alv} and $J_{NO,Br}$ did not differ significantly between subgroups of CF patients with and without chronic *Pseudomonas aeruginosa* infection (Fig. 3), as well as between CF patients with and without dornase α inhalation, even though the number

Table 1 Demographics of study population

	Healthy controls	Cystic fibrosis adults
N	20	12
Gender (female/male)	11/9	5/7
Age (range) (years)	27.4 (18.5–46.7)	24.2 (22.8–55.3)
FEV_1 (L)*	3.7 (3.2–5.5)	1.6 (0.9–3.6)
FEV_1 (% predicted)*	97 (83–118)	48 (26–89)
Chronic *Pseudomonas aeruginosa* infection	Not applicable	8
Nebulized dornase α long-term therapy	Not applicable	5

C_{Alv} = alveolar NO concentration; $J_{NO,Br}$ = bronchial nitric oxide flux; FEV_1 = forced expiratory volume in 1 s

Values are expressed as median (range)

* Mann–Whitney test $P < 0.0001$

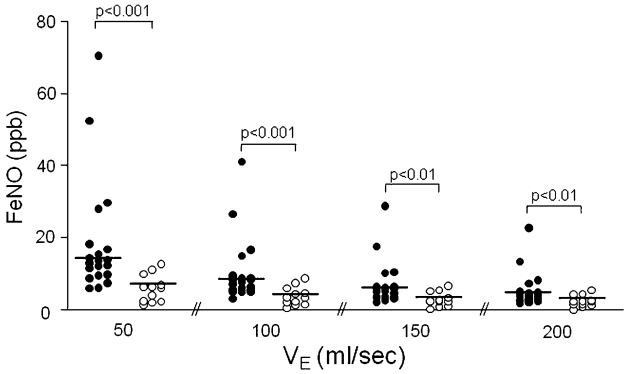

Fig. 2 End-expiratory FeNO at different fixed exhalation flow rates. The average end-expiratory FeNO from three consecutive measurements at each of four fixed exhalation flow rates ($V_E = 50$, 100, 150, and 200 ml/s) is shown for each healthy control (●) and each CF patient (○). The mean FeNO of each group is depicted as a line. Significant ($P < 0.05$) *P* values as calculated using the Mann–Whitney test are given above the groups compared

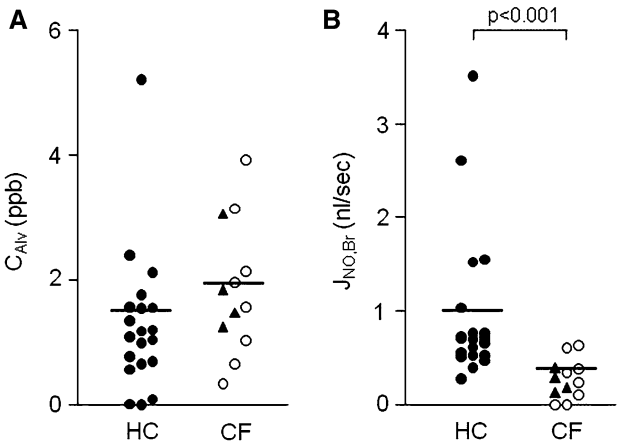

Fig. 3 Comparison of alveolar NO concentration (C_{Alv}) and bronchial NO flux ($J_{NO,Br}$) of healthy controls and CF patients. Alveolar NO concentrations (**a**) and bronchial NO fluxes (**b**) as calculated from the data presented in Fig. 2 are shown for each healthy control (●) and each CF patient without (○) and with (▲) chronic *Pseudomonas aeruginosa* infection. The mean value of each group is depicted as a line. Significant ($P < 0.05$) *P* values as calculated using the Mann–Whitney test are given above the groups compared

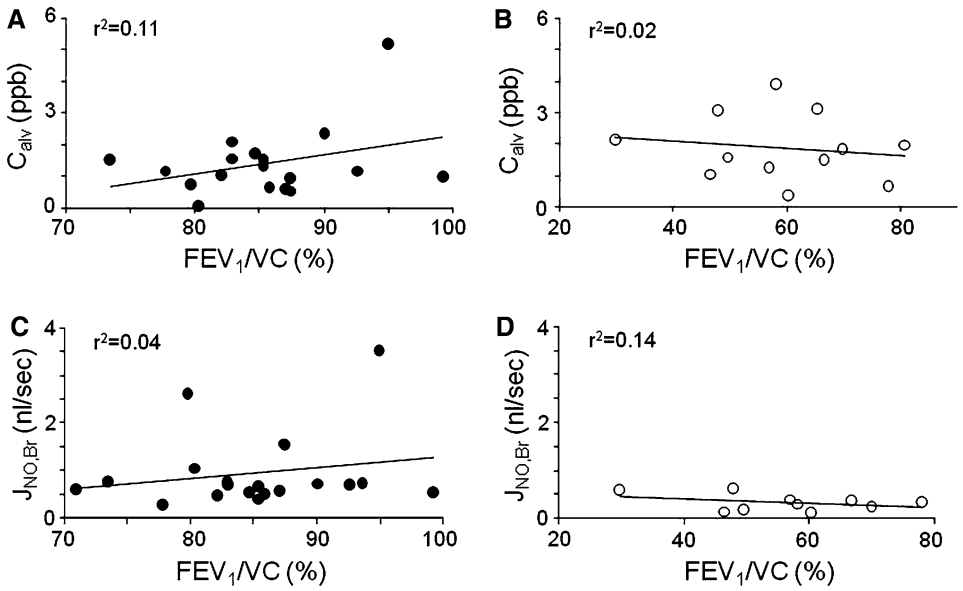

Fig. 4 Correlation between alveolar NO concentration (C_{Alv}) or bronchial NO flux ($J_{NO,Br}$) and airway obstruction (FEV/VC). The alveolar NO concentrations (**a**, **b**) and the bronchial NO fluxes (**c**, **d**) of healthy controls (●) and CF patients (○) were plotted against their corresponding airway obstruction as measured by spirometry immediately before FeNO measurements. Correlation was tested using Spearman's rank test

of patients in each subgroup was small [alveolar NO concentration: 1.8 ppb (95 CI = 0.8–2.8 ppb) vs. 1.9 ppb (95 CI = 0.7–3.1 ppb); bronchial NO flux: 0.426 nl/s (95 CI = 0.175–0.676 nl/s) vs. 0.229 nl/s (95 CI = 0.117–0.340 nl/s)].

Data of flow dependency of FeNO in CF patients and healthy control subjects are given in Fig. 4. No significant correlation was detectable between bronchial obstruction (FEV_1/VC) and the two independent NO exchange parameters C_{Alv} and $J_{NO,\,Br}$. Therefore, and after having proven the applicability of the two-compartment model to our data (Figs. 1 and 2), calculated C_{Alv} and $J_{NO,\,Br}$ values represent true parameters of NO exchange in the study patients and healthy controls.

Discussion

This study assessed flow-independent parameters of pulmonary NO exchange—the bronchial NO flux $J_{NO,Br}$ and the alveolar NO concentration C_{Alv}—in CF adults and healthy controls. Our data show that bronchial NO flux is lowered in adults with moderate CF lung disease. Furthermore, alveolar NO concentrations in healthy controls and CF adults were found to be similar. Moreover, no correlation was detected between bronchial airway obstruction and alveolar or bronchial NO in CF adults.

Nitric oxide is an important signalling mediator and is involved in multiple physiological and cellular functions in the lungs. Therefore, measurements of NO concentration in exhaled air have been utilized extensively for various airway diseases, in particular, asthma but also CF [1]. Measurements of FeNO have been performed successfully in infants, children, and adults. In CF, a common inherited disease in Caucasians, chronic neutrophilic inflammation of the lungs and chronic airway infection cause progressive lung disease, the leading cause of morbidity and mortality in CF patients [23]. However, despite the chronic inflammatory character of CF airways disease, FeNo has been shown to be rather normal or even decreased [8–10]. The suspected causes of decreased FeNO in CF are numerous and can be summarized as follows: (1) decreased NO production secondary to impaired iNOS expression/activity or L-arginine deficiency [24–29], (2) increased bacterial, enzymatic, or chemical NO consumption [4, 30–33], and (3) slower NO diffusion through the highly viscous and abundant mucus of CF patients [24, 34].

We found similar $J_{NO,Br}$ estimates for our CF patients with and without chronic *Pseudomonas aeruginosa* infection; therefore, NO consumption by resident bacterial pathogens or microbial enzymes seems unlikely the major cause for low bronchial NO output. In contrast, there was a trend toward higher bronchial NO flux in the subgroup of CF patients with dornase α treatment compared with those without dornase α treatment, most likely not statistically significant due to the small number of patients in this subgroup. High mucus viscosity presumably lowering NO diffusion rate and increasing net NO consumption during its passage through the mucus could thus be accountable for the low NO output in CF adults. In a recent placebo-controlled study, Grasemann et al. [35] showed that FeNO positively correlates with nebulized dornase α treatment in CF children. Nevertheless, based on our results and published evidence to date, it cannot be ruled out that impaired NO production also contributes to or even represents the major source of the measured deficit of exhaled NO in CF patients.

Flow-independent parameters of NO exchange have recently been explored. Shin et al. [36] used a single-breath technique that showed similar bronchial NO fluxes ($J_{NO,Br}$) but lower alveolar NO concentrations (C_{alv}) in CF children compared with healthy controls; however, the increase of mean NO was only weakly significant ($P = 0.05$), with a high standard deviation for CF patients and healthy controls. Suri et al. [18], using extended NO exchange measurements similar to the methods used in our study, reported that $J_{NO,Br}$ did not differ between healthy children and CF children while C_{alv} was higher in the patient group. The different results of the studies by Shin et al. and Suri et al. with respect to C_{alv} are likely due to the different experimental protocols and parameter estimation methods employed, since both studies enrolled patients of similar mean age (12 vs. 13.3 years) and mild CF lung disease (mean FEV_1/FVC ratios of 81 vs. 82). Our CF adult study cohort had a mean age of 30.3 years, more frequently chronic *Pseudomonas aeruginosa* infection, and moderate to severe lung disease. It remains to be proven if the degree of airway obstruction could explain the lowered bronchial NO fluxes in CF adults compared to the results of Shin et al. and Suri et al. Consequently, age-related features and the progression of CF lung disease might likely influence the bronchial NO flux in CF patients.

Suri et al. [18] reported that children with severe lung disease ($FEV_1 < 40\%$) were not able to perform exhalation maneuvers at highest flows. In contrast, our adult study patients with advanced CF lung disease experienced no particular problems using the same technique at higher flows. Adult age might be one of the reasons for this observation.

In our study, three-fourths of the CF patients were chronically infected with *Pseudomonas aeruginosa* and therefore treated long-term with oral azithromycin according to our center's policy. Azithromycin, a macrolide antibiotic, has frequently been used in recent years as an immunomodulatory agent in advanced CF lung disease, even though its mechanism of action remains unclear [37]. In our study, however, pulmonary NO exchange dynamics in CF adults with and without azithromycin therapy seem not to differ significantly; however, the patient numbers of each subgroup were too small to determine the impact of chronic airway infection and long-term azithromycin therapy reliably.

Our study has limitations which are part of its cross-sectional design and the small study cohort. Nevertheless, we were able to assess pulmonary NO exchange dynamics in CF adults, showing that CF patients without acute exacerbation have lower FeNO values compared to healthy controls as measured at exhalation flow rates of 50–200 ml/s. Alveolar NO concentrations are similar in CF and healthy adults, and the difference of FeNO is likely to be the result of lower bronchial NO flux. Because NO output is the sum of NO that originates from alveolar and bronchial compartments, our findings might explain the overall low values of FeNO in CF patients, probably influenced by multiple factors, including viscous mucus as a diffusion barrier for airway NO, NO consumption by bacterial enzymes, and decreased NO production.

Interestingly, four healthy controls in our study who did not have signs of atopy, asthma, or allergic rhinitis had high $FeNO_{50}$ ($\geq$30 ppb). However, even with their exclusion from the analysis, results remained similar and statistically significant.

The major goal of our study was a proof of principle, that is, the validity of the methodology proposed by Tsoukias and George to assess alveolar and bronchial contributions to exhaled NO in CF adults. The described method using three additional flow rates of 100, 150, and 200 ml/s can be performed easily and allows the calculation of alveolar NO concentration (C_{Alv}) and bronchial NO flux ($J_{NO,Br}$) with a simple linear regression equation.

Further work is needed to fully understand pulmonary NO exchange dynamics in the different stages of CF lung disease across different age groups and in larger cohorts, but also compared to other chronic lung diseases such as non-CF bronchiectasis.

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