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Increased nitrotyrosine in exhaled breath condensate in cystic fibrosis

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ABSTRACT: Exhaled nitric oxide (ENO), a marker of inflammation in airway diseases is decreased in cystic fibrosis (CF) patients, perhaps because nitric oxide (NO) is metabolized to oxidative end-products. A stable product, 3-nitrotyrosine, may indicate local formation of reactive nitrogen species.

Whether NO metabolites in exhaled breath condensate may be increased in CF patients was investigated. The fractional concentration of ENO (F_{eno}), nitrotyrosine and oxides of nitrogen in exhaled breath condensate from 36 stable CF patients were compared to 14 normal subjects using an enzyme immunoassay and fluorescence assay.

Nitrotyrosine levels in breath condensate were increased significantly in stable CF patients, compared with normal subjects (25.3 ± 1.5 *versus* 6.3 ± 0.8 ng·mL^{-1}, $p < 0.0001$). There was an inverse correlation between the levels of nitrotyrosine and the severity of lung disease. F_{eno} levels were significantly lower in CF patients than in normal subjects (4.4 ± 0.3 versus 5.6 ± 0.4 (parts per billion), $p < 0.05$). No correlation was found between nitrotyrosine and F_{eno} levels in CF. There was no significant difference in the levels of nitrite and nitrate between CF patients and normals.

The elevation in nitrotyrosine may reflect increased formation of reactive nitrogen species such as peroxynitrite or direct nitration by granulocyte peroxidases, indicating increased oxidative stress in airways of cystic fibrosis patients.
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Nitric oxide (NO) is a gas produced from L-arginine by NO synthases (NOS) [1], of which three isoforms have been identified [2]. NO has an important role in several physiological processes in the respiratory tract, including vascular regulation, neurotransmission, host defense, and cytotoxicity [3, 4]. In inflammatory lung diseases such as asthma and bronchiectasis, NO production is increased, probably due to the induction of inducible NOS (iNOS) [5–6].

Cystic fibrosis (CF) is characterized by recurrent pulmonary infections and increased oxidative stress leading to damage of airways. However, despite chronic airway inflammation in CF, the fractional concentration of exhaled NO (F_{eno}) is decreased in stable CF patients [7, 8]. There are several possible explanations for the paradoxical reduction in F_{eno}, such as reduced expression of iNOS in CF [9], and an increased metabolism of NO to reactive nitrogen intermediates, such as nitrite (NO_2^-), nitrate (NO_3^-), s-nitrosothiols and peroxynitrite ($ONOO^-$) [8]. The microenvironment of the CF lung – (viscous mucus secretion and increased reactive oxygen species release from inflammatory cells) may facilitate the reaction of NO with inflammatory oxidants causing an increased formation of reactive NO metabolites.

Nitrotyrosine has been considered to be an indicator of the involvement of reactive nitrogen species [10]. In the airway epithelium and inflammatory cells of patients with asthma there is a strong immunoreactivity for nitrotyrosine, suggesting a pathophysiological role for reactive nitrogen species in inflammatory lung diseases [11]. Nitration of proteins is a biological process derived from the biochemical interaction of NO or NO-derived secondary products with reactive oxygen species. Multiple pathways under different conditions can mediate tyrosine nitration although not all nitrating pathways are relevant *in vivo* [12]. Peroxynitrite can nitrate the tyrosine residues of proteins to yield the stable product 3-nitrotyrosine. In chronic inflammation, or other inflammatory cell-mediated processes, a myeloperoxidase (MPO)-dependent pathways also contribute to the formation of tyrosine nitration, since MPO from polymorphonuclearneutrophils converts

nitrite into NO_2Cl and NO_2, resulting in tyrosine nitration [10, 12].

NO metabolites, such as NO_2^-, and NO_3^-, can be detected in the epithelial lining fluid of the normal human respiratory tract as well as in exhaled breath condensate [8], and probably reflects the NO metabolism in CF more than F_{eNO} [8].

This study assessed the levels of 3-nitrotyrosine, NO_2^- and NO_3^- in exhaled breath condensate, and F_{eNO} and lung function were assessed in clinically stable CF patients, compared with normal subjects. The aim of this study was to explore a noninvasive technique for measurement of oxidative stress in the lungs of patients with CF.

Materials and methods

Patients

Patients were recruited from the adult CF clinic at the Royal Brompton Hospital, London UK. Those colonized with *Burkholderia cepacia*, methicillin resistant *Staphylococcus aureus*, or with an acute chest infection or disease exacerbation were excluded from the study. The criteria of clinical stability were: no increased cough and/or increased sputum production, no change in quality of sputum (more, purulent, increased "thickness", or presence of blood), no sensation of increased dyspnoea, no decreased exercise tolerance or worsening of forced expiratory volume in one second FEV1. Thirty-six patients with CF (23 male, aged 29 ± 1 yr, FEV1 59.7 ± 26.4% predicted, (range of FEV1 % pred is 110.4), 16 of whom were receiving inhaled and/or oral corticosteroids, were studied. All patients were life-long nonsmokers. For all patients sputum culture and blood test were performed. Twenty-two (61%) were positive for *Pseudomonas aeruginosa* ($>10^5$ cfu·mL^{-1}). Twelve (33%) had other pathogens (*S. aureus*, *Haemophilus influenzae*) and two sputum cultures had normal flora. Five (14%) sputum cultures were positive for both *P. aeruginosa* and *S. aureus*. Circulating leucocyte count (WBC) was 10.5 ± 0.5 g·L^{-1}, and sedimentation was 18.3 ± 3.5 mm·h^{-1}. The control group consisted of 14 nonsmoking healthy volunteers (six male, 34 ± 2 yrs, FEV1 101.8 ± 2.6% pred). None of the controls had a history of respiratory or cardiovascular disease, or were receiving any medication. The ages of the groups were not statistically different. Clinical characteristics of the subjects are displayed in table 1.

The study protocol was approved by the Ethics Committee of the Royal Brompton Hospital, and informed consent was obtained from each subject.

Pulmonary function

Forced vital capacity (FVC) and FEV1 were measured with a dry spirometer (Vitalograph, Buckingham, UK) and the best value of three manoeuvres was expressed as a percentage of the predicted value. Measurement of total lung capacity (TLC) and residual volume (RV) was performed by body plethysmography. (Transfer Factor Model B; PK Morgan; Rainham, UK).

Exhaled nitric oxide measurement

F_{eNO} was measured by chemiluminescence analyser (Model LR2000, Logan Research Ltd, Rochester, UK); sensitivity to NO from 1 – 500 parts per billion (ppb) by volume, and with resolution of 0.3 ppb. The analyser was designed for online recording of F_{eNO} concentrations. It was calibrated with certified NO mixtures (55 ppb) in nitrogen (BOC Special gases, Guilford, UK). Measurement of F_{eNO} was made by slow exhalation (5 – 6 L·min^{-1}) from TLC for 20 – 25 s against a resistance (3 ± 0.4 mmHg), to prevent nasal contamination. The mean values were taken from the point corresponding to the plateau of end-exhaled CO_2 reading, representing the lower respiratory tract sample.

Exhaled breath condensate

Exhaled breath condensate was collected using a condenser, which allowed the noninvasive collection of nongaseous components of the expiratory air (EcoScreen, Jaeger, Würzburg, Germany). After rinsing their mouths, subjects breathed through a

Table 1. – Patient characteristics

	CF not steroid-treated	CF steroid-treated	Normal Subjects
Subject n	20	16	14
Age yrs	27 ± 2	30 ± 2	34 ± 2
Sex M/F	14/6	9/7	6/8
FVC % pred	86.3 ± 4.9	74.7 ± 5.5	100.5 ± 3.1
FEV1 % pred	67.5 ± 6.5	51.9 ± 4.9	101.8 ± 2.6
P. aeruginosa in sputum culture $>10^5$ cfu·mL^{-1}	9	13	0
Therapy:			
Inhaled steroids	0	9	0
Oral steroids	0	5	0
Oral + inhaled steroids	0	2	0

Data are presented as mean ± SEM or numbers of objects. CF: cystic fibrosis patients; M: male; F: female; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; PA: *Pseudomonas aeruginosa*.

mouthpiece and a two-way nonrebreathing valve, which also served as a saliva trap. They were asked to breathe at a normal frequency and tidal volume, wearing a noseclip, for a period of 10 min. The condensate, at least 1 mL, was collected as ice at -20 °C and stored at -70 °C immediately.

Nitrite and nitrite plus nitrate measurement

Quantification of NO_2^- was assessed by a fluorometric assay based upon the reaction of NO_2^- with 2, 3-diaminonaphthalene (DAN) to form the fluorescent product, 1-(H)-naphthotriazole [13]. The lower limit of detection for this assay was 0.1 μM. Briefly, a 100-μL sample (exhaled breath condensate) was mixed with 10 μL of 0.05 mg·mL DAN reagent in 0.625 M HCl. The reaction was allowed to proceed at room temperature in the dark and was terminated with 10 μL of 1.4 M NaOH. The intensity of the fluorescent signal produced by the product was immediately measured by a fluorometer (Excitatory wavelength: 360 nm, Emmission wavelength: 460 nm: Biolite F1, Labtech, International Ltd, Uckfield, UK). Incubation of samples with NO_2^- reductase, allowed the NO_3^- present in the sample to be measured by this assay after being converted to NO_2^-.

Nitrotyrosine assay

Nitrotyrosine was measured with a specific enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI, USA). The assay has been validated to obtain a high correlation (0.95) between added known amounts of nitrotyrosine and the concentration measured by EIA. The kit has been used to measure nitrotyrosine concentrations in breath condensate. The lower limit of detection for this assay was 3.9 $ng \cdot mL^{-1}$. The samples were concentrated threefold by a 12-h dryfreeze procedure (Modulyo, Edwards, Crawley, UK) and nitrotyrosine was assessed by the EIA method. The actual concentration of nitrotyrosine was calculated back according to the concentration procedure.

The possible influence of the ventilation rate on nitrotyrosine concentrations in breath condensate was assessed. Normal volunteers breathed at 14 and 28 $breaths \cdot min^{-1}$ for 15 min, maintaining the same tidal volume. There was no difference in the levels of nitrotyrosine in the two samples collected from the same subject at the different ventilation rates (6.4 $ng \cdot mL^{-1}$ and 7.1 $ng \cdot mL^{-1}$ $n=6$).

To assess the repeatability of the test procedure, 21 samples from seven subjects (triplicates) were measured (variation coefficient of intraassay was 5%), samples collected in three consecutive days from the same seven subjects were also measured (day to day repeatability) and the coefficient of variation was 6%. Amylase was undetectable in six samples tested, ruling out saliva contamination of breath condensate by a photometrical method (BM/Hitachi 917, Japan).

Statistical analysis

Data were expressed as means ± SEM. Statistical comparisons between groups were performed using a t-test. The correlation between F_{ENO} and nitrotyrosine level, as well as nitrotyrosine level and lung function (FVC, FEV_1, RV, TLC) was determined by Pearson correlation coefficient and multiple correlation analysis. Discriminant function analysis was performed to compare the steroid and nonsteroid treated groups. Significance was defined as when $p<0.05$.

Results

Exhaled nitric oxide

F_{ENO} levels significantly decreased in CF patients compared to normal subjects (4.4 ± 0.3 *versus* 5.6 ± 0.4 ppb, $p<0.05$) There was no significant difference between steroid-naive and steroid-treated groups (4.7 ± 0.4 *versus* 4.1 ± 0.6 ppb) (fig. 1). There was no correlation between F_{ENO} and lung function parameters (FVC, FEV_1, RV/TLC) of CF patients or between white blood cell (WBC), sedimentation and F_{ENO}.

Nitrotyrosine in exhaled breath condensate

Nitrotyrosine concentrations were detectable in breath condensate of normal subjects (6.3 ± 0.8 $ng \cdot mL^{-1}$) and were increased significantly in clinically stable CF patients (25.3 ± 1.5 $ng \cdot mL^{-1}$, $p<0.0001$) (fig. 2). There was no significant difference in nitrotyrosine levels between the steroid-naive and steroid-treated groups (25.5 ± 1.9 *versus* 24.2 ± 2.5 $ng \cdot ml^{-1}$). There was no significant correlation between F_{ENO} and nitrotyrosine levels in CF patients. To assess whether the severity of lung disorders in CF influenced

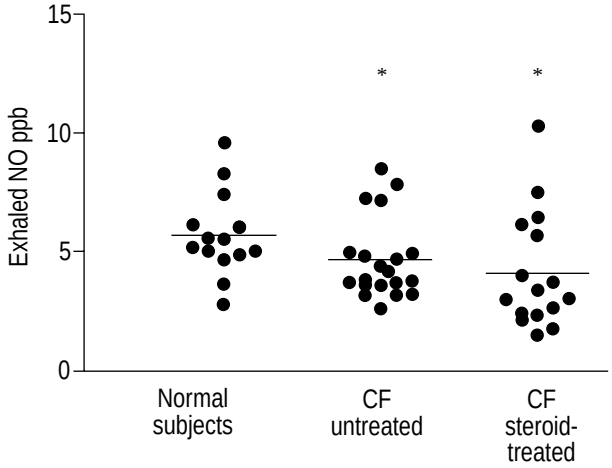

Fig. 1. – Nitric oxide (NO) in exhaled air of normal subjects and patients with cystic fibrosis (CF) who were and were not treated with inhaled/oral steroids. Median levels (horizontal bars) in subjects with CF were significantly lower than normal controls, regardless of usage of inhaled steroids (*: $p<0.05$). No significant difference was found between CF groups. ppb: parts per billion.

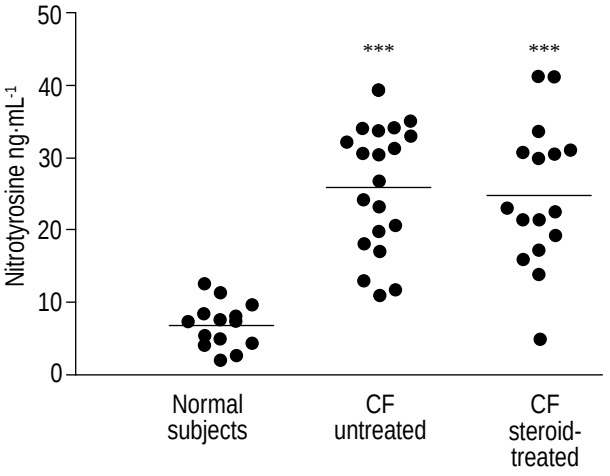

Fig. 2. – Nitrotyrosine in exhaled breath condensate of normal subjects and patients with cystic fibrosis (CF) who were and were not treated with inhaled oral steroids. Median levels (horizontal bars) in subjects with CF were significantly higher than normal controls regardless of usage of inhaled steroids (***: $p<0.001$).

nitrotyrosine concentrations, the relationship between nitrotyrosine in exhaled breath condensate and pulmonary function in CF patients was investigated. A positive correlation was observed between nitrotyrosine and FVC % pred ($r=0.71$, $p<0.0001$, linear regression analysis), FEV_1 % pred ($r=0.70$, $p<0.0001$). RV/TLC % pred correlated inversely to the nitrotyrosine level ($r=-0.70$, $p<0.0001$) indicating that, better lung function was associated with higher nitrotyrosine concentrations (fig. 3). The multiple regression coefficient ($R=0.73$) was significant ($p<0.0001$). There was no significant difference in nitrotyrosine levels between CF patients with a positive *P. aeruginosa* sputum culture compared to CF patients infected with other pathogens (25.4 ± 1.8 *versus* 23.8 ± 2.7 ng·mL^{-1}). The mean difference was not statistically significant. WBC, sedimentation, and neutrophil per cent of WBC were assessed. As multiple regression analysis proved, there was no correlation between these parameters and nitrotyrosine level.

Nitrite and nitrite plus nitrate in breath condensate

No significant difference was observed in the levels of NO_2^- and NO_2^- plus NO_3^- in exhaled breath condensate between patients with CF and healthy subjects (3.9 ± 0.7 *versus* 3.2 ± 0.5 μM; 25.4 ± 6.1 *versus* 21.9 ± 3.2 μM). There was no correlation between NO_2^-, NO_2^- plus NO_3^- and nitrotyrosine levels in exhaled breath condensate. Analysing the multiple regression relationship between NO_2^- and lung function as well as NO_2^- plus NO_3^- and lung function (FEV_1 % pred, FVC % pred, RV/TLC % pred) no significant association was found.

Discussion

This study investigated whether nitrotyrosine in exhaled breath condensate was a marker of airway inflammation in patients with CF. The findings demonstrate that nitrotyrosine in exhaled breath is significantly higher in stable CF than in nonsmoking healthy controls.

Nitrotyrosine is a marker of protein nitration and can be detected by using a specific nitrotyrosine

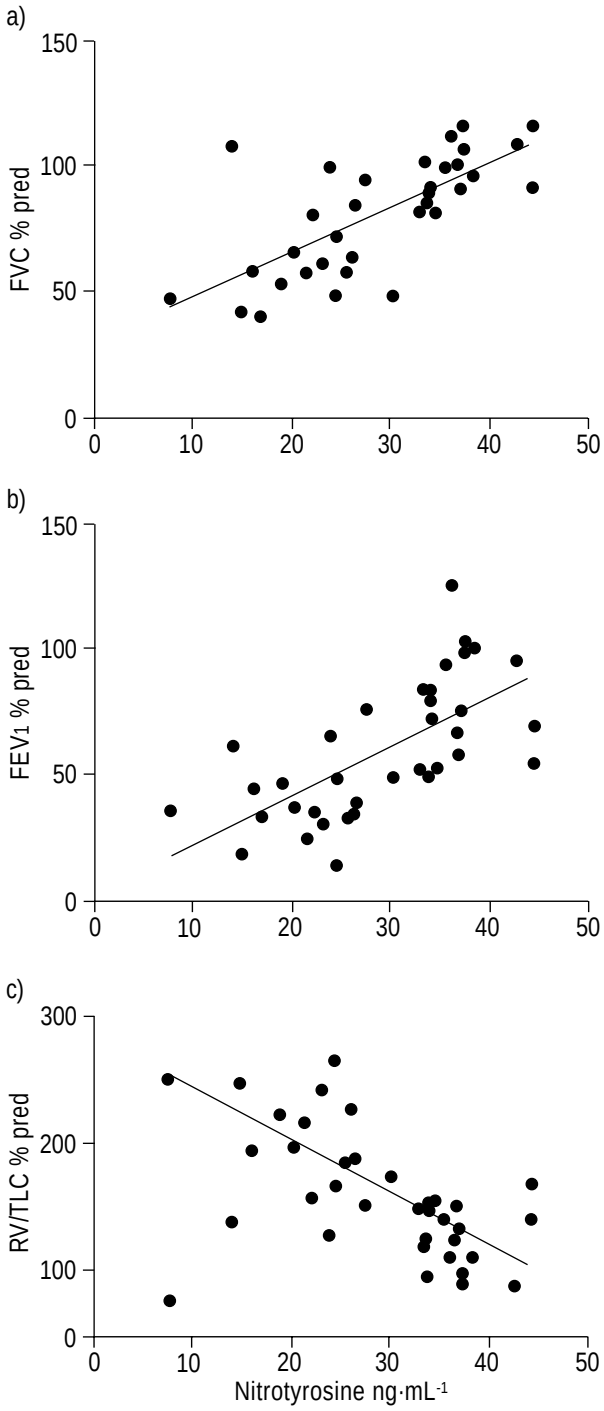

Fig. 3. – Correlations (Pearson) between percentage predicted a) forced vital capacity (FVC; $r=0.71$, $p<0.0001$); b) forced expiratory volume in one second (FEV_1; $r=0.70$, $p<0.0001$); c) residual volume/total lung capacity (RV/TLC; $r=-0.70$, $p<0.0001$) and nitrotyrosine in exhaled breath condensate, of all cystic fibrosis patients measured on the same day.

antibody. Nitration of proteins is a biological process derived from the biochemical interaction of NO or NO-derived secondary products with reactive oxygen species [12, 14]. Tyrosine nitration can be mediated by multiple pathways under different conditions, suggesting that nitrotyrosine may be considered as a collective indicator for the involvement of reactive nitrogen species [10]. Overall, several nitrating agents can satisfy the requirements for biological nitration, although presently, limited information is available regarding the relative effectiveness of the nitrating species to nitrate tyrosine residues *in vivo* [12]. Nitration of tyrosine could impact deleteriously on cellular function and viability because this specific modification is known to alter protein function *in vitro* [10].

Peroxynitrite is a potent oxidant, formed by the rapid reaction of the free radicals NO and O_2^- and causes tyrosine nitration in lung tissue [12, 15]. It can initiate lipid peroxidation in biological membranes, hydroxylation and nitration of aromatic amino acid residues and sulphydril oxidation of proteins [2–4, 12, 16–17]. It can be converted to peroxynitrous acid (ONOOH) with subsequent cleavage and release of an intermediate with hydroxyl radical (·OH)-like activity [15]. The toxicity of peroxynitrite is due to the direct reactions of the anion ($ONOO^-$), as well as reactivities of the acid (ONOOH). Peroxynitrite induces hyperresponsiveness in the airways of guinea pigs [18], inhibits pulmonary surfactant [19], damages pulmonary epithelial cells [16], and oxidizes glutathione [17]. Increased production of peroxynitrite has been reported in the airways and lung parenchyma in several lung diseases, associated with chronic inflammation and/or oxidative stress [20–21]. Activation of inflammatory cells, such as neutrophils, eosinophils and macrophages induce a marked production of O_2^- facilitating the formation of peroxynitrite [22].

In chronic inflammation, or other inflammatory cell-mediated process, the myeloperoxidase (MPO)-dependent pathways must be considered. Large numbers of polymorphonuclear neutrophils (PMN) accumulate in airways of CF patients, and lead to increased MPO activity [23]. Activated human PMNs can convert NO_2^- into inflammatory oxidants through the MPO pathway [10]. It has been suggested that MPO-catalysed nitration in the presence of hydrogen peroxide (H_2O_2) to form nitrating intermediates from NO_2^-, a main end-product of NO, is an alternative mechanism of protein nitration, which is independent of peroxynitrite [10].

The other pathways of the formation of nitrotyrosine which are detected *in vitro*, including direct oxidation of NO_2^- by hydrogen peroxide or hypochlorous acid or reaction of NO or nitrogen dioxide (NO_2) with tyrosyl radicals *in vivo* have not been completely elucidated [12]. Recent data reveals some clinical conditions result in endogenous airway acidification, which can modulate NO biochemistry [24]. pH can play a role in the formation of nitrotyrosine, but further studies are necessary to clarify this point *in vivo*.

Recent publication has demonstrated an increased level of NO_3^- and nitrotyrosine as well as myeloperoxidase, but not NO_2^- in CF sputa suggesting increased production of NO in the lower respiratory tract of CF patients [25]. CUNNINGHAM *et al.* [26] reported an elevated NO_2^- level in exhaled breath condensate in CF patients supposing that NO_2^- is the result of NO degradation within CF mucus. Another study has found no elevated levels of NO_2^- and NO_3^- but increased level of myeloperoxidase in CF sputa [27].

In agreement with the latest study [27] no significant difference was observed in NO_2^- or NO_2^- plus NO_3^- levels in breath condensate between CF and normal subjects. The increased level of nitrotyrosine in exhaled breath condensate has overlapped with the elevated level of nitrotyrosine in CF sputa (26). This may indicate that most nitrotyrosine formation is through MPO *via* increased neutrophil infiltration. This study provided evidence that oxidative stress induced by inflammation produces nitrotyrosine, which presumably reflects increased direct nitration by granulocyte peroxidases.

There was a significant reduction in F_{eno} in patients with CF compared with normal subjects, as previously reported [7, 8, 28]. It has been explained that a significant proportion of NO from the lower airways may have been metabolized by oxidation to biologically active nitrogen oxides before reaching the air spaces [7, 8]. The finding of a marked increase in nitrotyrosine suggests it is possible that NO metabolites in the CF airways may be metabolized to peroxynitrite. However, no increase was detected in NO_2^- plus NO_3^- which may be expected if peroxynitrite is increased suggesting the priority of another pathway (MPO-pathway) of nitrotyrosine formation in CF airways. There was no significant difference in F_{eno} between CF patients colonized with only *P. aeruginosa* compared to those colonized with other species, in agreement with a recent publication by THOMAS *et al.* [28].

In this study no significant difference was found in the F_{eno} or nitrotyrosine in breath condensate between patients treated or not treated with steroids. Steroid therapy is the mainstay of treatment in asthma [5, 29], but remains controversial in CF [30]. Nitrotyrosine formation in the airways, which is not influenced by steroid treatment in CF patients, may reflect a different type of inflammation that is not steroid sensitive, in the same way that the inflammation of chronic obstructive lung disease (COPD) fails to be suppressed by steroid therapy [31].

A strong inverse correlation was demonstrated between the level of nitrotyrosine and the severity of the lung disease measured by lung function. There are conflicting data regarding the correlation of NO oxidative metabolites and lung function in CF. GRASEMANN *et al.* [7] found a positive correlation between F_{eno} concentration and FVC, but not FEV_1, indicating that FENO is inversely related to disease severity [7] and also reported that NO metabolites in sputum correlated positively with FEV_1 as well as FVC [32]. In contrast, HO *et al.* [8] found no correlation between NO_2^- in exhaled breath condensate and lung function. The positive correlation between nitrotyrosine in exhaled breath and the lung function parameters suggests that higher nitrotyrosine

levels reflect higher NO production in airways of CF patients with milder disease. Probably, like F_{eno} in patients with advanced disease, the damage of lung parenchyma may contribute to the decreased NO production. Although no correlation could be detected between nitrotyrosine levels in breath condensate and F_{eno} and NO metabolites (NO_2^-, NO_2^- plus NO_3^-) which suggests the possibility of nitrotyrosine formation rather through the MPO pathway. The other possible explanation is that the impaired ventilation in small bronchi due to increased amount of bronchial secretion, destruction of airway wall and increased anatomical dead space [33], in advanced disease, may inhibit the release of free nitrotyrosine from the lower airways into exhaled air. Yet another explanation might be the differences in the lung clearance of nitrotyrosine. In the presence of severe ventilation inhomogenities, the clearance of nitrotyrosine might be altered. Furthermore, pH may play a role in the formation of nitrotyrosine. If more severe disease were associated with a more acidic pH, peroxynitrite would be more likely to dissociate into hydroxyl and nitrogen dioxide and less likely to react with tyrosine radicals yielding the stable 3-nitrotyrosine.

On the other hand, several studies have suggested that NO might act as an antioxidant to counteract the cytotoxic effects of reactive species [34]. Supposing that increased nitrotyrosine levels are associated with milder disease reflecting higher NO production, it may protect the lung against injury by the reactive oxidant radicals.

In summary, this study provided evidence for increased production of nitrotyrosine in patients with CF, which may reflect increased formation of reactive nitrogen species such as peroxynitrite or direct nitration by granulocyte peroxidases. Nitrotyrosine, which is formed in the airways and can be collected in the exhaled breath condensate, may be a marker of oxidative stress in CF patients. Further studies are necessary to clarify the complex chemistry of NO and its oxidative products, such as peroxynitrite in chronic suppurative lung diseases.

The elevation in nitrotyrosine may reflect increased formation of reactive nitrogen species such as peroxynitrite or direct nitration by granulocyte peroxidases, indicating increased oxidative stress in airways of cystic fibrosis patients.

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