

## CLINICAL STUDY

# Nitrites and nitrates in exhaled breath condensate in cystic fibrosis: relation to clinical parameters

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**Abstract:** *Objectives:* To evaluate correlation of exhaled breath condensate (EBC) nitrite and nitrate concentrations with disease severity in cystic fibrosis (CF) patients.

*Background:* Nitrites and nitrates are products of oxidative metabolism of nitric oxide. Impaired metabolism of nitric oxide plays a role in pathogenesis of CF.

*Methods:* EBC was collected from 46 stable CF patients and from 21 healthy controls. EBC concentrations of nitrites and nitrates were correlated with parameters of lung disease and nutritional status and with systemic inflammatory markers.

*Results:* EBC nitrates concentrations in CF patients were lower than in healthy subjects (5.8 vs 14.3  $\mu\text{mol/l}$ ,  $p < 0.001$ ). They correlated positively with  $\text{FEV}_1$  ( $p = 0.025$ ) and serum albumin values ( $p = 0.016$ ) and negatively with chest radiograph Northern score ( $p = 0.015$ ) and serum C-reactive protein values ( $p = 0.005$ ). EBC nitrites concentrations in CF patients did not differ from those in healthy subjects and were not correlated to any studied parameter.

*Conclusions:* EBC nitrates concentrations correlate with disease severity in CF patients and are lower than in healthy subjects (Tab. 4, Fig. 1, Ref. 48). Full Text in PDF [www.elis.sk](http://www.elis.sk).

**Key words:** cystic fibrosis, disease severity, exhaled breath condensate, nitrites, nitrates.

**Abbreviations:** BMI – body mass index, CF – cystic fibrosis, CRP – C-reactive protein, EBC – exhaled breath condensate, eNO – exhaled nitric oxide,  $\text{FEV}_1$  – forced expiratory volume in first second, IgG – immunoglobulin G, iNOS – inducible nitric oxide synthase.

Bronchopulmonary disease in cystic fibrosis (CF) is characterised by impaired mucociliary clearance, chronic bacterial infection and neutrophilic inflammation (1). Beside the dysfunction of cystic fibrosis transmembrane conductance regulator (CFTR), an impaired nitric oxide (NO) metabolism plays a role in the pathogenesis of CF (2). The values of exhaled NO (eNO) in CF adults

are lower when compared with healthy subjects and are positively correlated with pulmonary function (3, 4, 5).

Nitrites ( $\text{NO}_2^-$ ) and nitrates ( $\text{NO}_3^-$ ) are products of oxidative metabolism of NO. The studies measuring  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in sputum or bronchoalveolar lavage fluid have found either unchanged or increased concentrations of these metabolites in patients with CF compared to healthy subjects (6, 7, 8, 9). The concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in sputum correlated positively with pulmonary function (6).

Exhaled breath condensate (EBC) is collected by cooling of expired gas during tidal breathing. This is a non-invasive and easy-to-perform method of sampling the secretions from airways (10). The concentrations of  $\text{NO}_2^-$  in EBC in CF patients were similar or higher when compared with healthy controls and did not correlate with pulmonary function (11, 12). On the other hand, the concentrations of  $\text{NO}_3^-$  in EBC were similar or lower in CF patients than in controls (13, 14, 15).

In the present study, the relationships between concentrations of NO metabolites and clinical characteristics of CF patients were analysed by using a commercial device for EBC collection and a thoroughly validated analytical method for measurement of EBC concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in a large study population.

## Methods

Patients with CF were consecutively recruited into the study at the Pulmonary Department of Charles University, 2nd Faculty of Medicine, and University Hospital Motol in Prague, Czech Republic.

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lic. The diagnosis of CF was confirmed in all patients by repeated sweat test with chloride concentration exceeding 60 mmol/l and by mutation analysis of *CFTR* gene. Respiratory disorders other than CF and smoking history were the main exclusion criteria.

The examinations were performed during routine out-patient visits in a stable phase of the disease. Demographic parameters (sex and age) and sputum microbiology (predominant bacteria) were collected from patients' records. Written informed consent was obtained from all subjects. The study was approved by Ethical Committee of Charles University, 2nd Faculty of Medicine, Prague, Czech Republic. Control healthy subjects were staff members or students with no history of smoking or any chronic lung disease.

EBC collection was performed according to ERS/ATS guidelines using ECoScreen device (Jaeger, Germany) (10). Obtained EBC was frozen immediately and stored at  $-80^{\circ}\text{C}$  until examined. EBC  $\text{NO}_2^-$  and  $\text{NO}_3^-$  concentrations were assayed by liquid chromatography after derivatization with diaminonaphthalene, as described previously (16). EBC collection was carried out in antemeridian hours and was preceded by chest physiotherapy early in the morning with nebulized dornase alpha. Lung function tests were completed after the collection of EBC.

Lung function tests were performed using Jaeger MasterLab device (Jaeger, Germany) according to ERS/ATS guidelines with reversibility testing (with 400  $\mu\text{g}$  of salbutamol) (17). Better results from pre-/postbronchodilatory values of forced expiratory volume in the first second ( $\text{FEV}_1$ ) were reported. Chest radiographs were

done and scored using the Northern score (18). Body weight and body height were measured under standard conditions and body mass indexes (BMI) were calculated. Blood for routine biochemical analyses was sampled by venepuncture under standard conditions. Routine biochemical analyses, i.e. serum C-reactive protein (CRP), immunoglobulin G (IgG) and albumin were performed using standard techniques.

Data were expressed as arithmetic or geometric means (for normally or log-normally distributed data, respectively) and 95 % confidence intervals (CI) for means. Comparisons between groups were made using Fisher's exact test and two-sample t-test. The values were log-transformed when the distribution of original variables was highly skewed ( $\text{CRP}$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ). Evaluation of correlations between variables was made using Spearman's rank correlation coefficient ( $r_s$ ). A p-value  $<0.05$  was considered statistically significant. Statistical software Stata, release 9 (Stata-Corp LP, College Station, TX) was used for analysis.

## Results

Forty-six patients with CF and 21 healthy subjects were enrolled in the study. No differences were found in the demographic data between these two groups. Gram-negative bacteria, i.e. *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex, predominated in sputum cultures in 40 patients (Tab. 1).

Clinical characteristics of CF patients and results of laboratory tests are presented for the whole group as well as separately for females and males. No sex-related differences were found except for lower serum albumin values in females (Tab. 2).

Concentrations of  $\text{NO}_3^-$  were lower in CF patients than in healthy subjects, while the levels of  $\text{NO}_2^-$  did not differ between these groups. Sex did not influence the EBC NO metabolites concentrations in CF patients (Tab. 3).

No significant correlation was found between EBC  $\text{NO}_2^-$  concentration and any of clinical parameters in CF patients. The  $\text{NO}_3^-$  values were correlated with several clinical characteristics. The  $\text{NO}_3^-$  concentration correlated positively with  $\text{FEV}_1$  value and serum albumin concentration. Negative correlation was observed between  $\text{NO}_3^-$  concentration and Northern score and also with serum CRP concentration (Tab. 4). Significant correlations are depicted in Figure 1.

**Tab. 1. Demographic data in CF patients and in healthy controls.**

Parameter	CF patients (n=46)	Healthy controls (n=21)	p-value
Age (years)	24.6 (19.5–32.9)	26.0 (15.0–38.0)	0.246*
Sex (M / F)	23 / 23	12 / 9	0.610 <sup>§</sup>
Sputum microbiology			
no bacteria	1 (2.1%)		
Styphlococcus aureus	5 (10.9%)		
Pseudomonas aeruginosa	12 (26.1%)		
Burkholderia cepacia complex	28 (60.9%)		

Age is expressed as arithmetic mean and 95% CI; \* two-sample t-test; <sup>§</sup>Fisher's exact test

**Tab. 2. Clinical characteristics in CF patients**

Parameter	CF patients (n=46)	Sex-specific characteristics in CF patients		
		Males (n=23)	Females (n=23)	p-value*
$\text{FEV}_1$ (% pred.)	59.1 (53.2-65.0)	58.4 (50.8-66.0)	59.8 (50.3-69.4)	0.810
Northern score	8.0 (7.1-8.8)	8.1 (6.9-9.3)	7.9 (6.6-9.2)	0.802
BMI ( $\text{kg}/\text{m}^2$ )	20.8 (20.1-21.6)	20.7 (19.7-21.8)	20.9 (19.8-22.0)	0.779
Albumin (g/l)	42.0 (41.4-42.9)	43.3 (42.1-44.5)	40.7(39.5-41.9)	0.003
CRP (mg/l)	8.3 (5.9-11.7)	8.4 (5.0-13.9)	8.2 (5.0-13.6)	0.961
IgG (g/l)	18.0 (16.6-19.5)	17.2 (15.0-19.4)	18.9 (16.9-20.9)	0.249

Values are expressed as arithmetic means ( $\text{FEV}_1$ , Northern score, BMI, albumin and IgG) or geometric means (CRP) and 95% CI; \* two-sample t-test

**Tab. 3. EBC concentrations of nitrites and nitrates in CF patients and in healthy controls**

Parameter	Healthy controls (n=21)	CF patients (n=46)	p-value*	Sex-specific characteristics in CF patients		
				Males (n=23)	Females (n=23)	p-value*
Nitrites ( $\mu\text{mol}/\text{l}$ )	4.3 (3.7-4.9)	4.4 (3.5-5.5)	0.853	5.1 (3.9-6.8)	3.8 (2.7-5.3)	0.159
Nitrates ( $\mu\text{mol}/\text{l}$ )	14.3 (10.7-19.3)	5.8 (4.2-7.8)	$<0.001$	6.2 (4.0-9.5)	5.3 (3.3-8.6)	0.632

Values are expressed as geometric means and 95% CI; \* two-sample t-test

**Tab. 4. Spearman's correlations ( $r_s$ ) between clinical characteristics of CF patients and EBC concentrations of nitrites and nitrates.**

Parameter	Nitrites ( $\mu\text{mol/l}$ )		Nitrates ( $\mu\text{mol/l}$ )	
	$r_s$	p-value	$r_s$	p-value
FEV <sub>1</sub> (% pred.)	0.14	0.331	0.33	0.025
Northern score	-0.09	0.565	-0.36	0.015
BMI (kg/m <sup>2</sup> )	0.05	0.738	0.27	0.068
Albumin (g/l)	0.12	0.423	0.35	0.016
CRP (mg/l)	-0.09	0.533	-0.40	0.005
IgG (g/l)	-0.20	0.188	-0.21	0.089

**Discussion**

CF is generally considered to be a disease with decreased eNO. The recent publications report eNO in CF to be similar (19, 20) or lower (21, 22, 23) than in controls. Positive correlation with FEV<sub>1</sub> value (3, 5, 24) or disease severity (Shwachman-Kulczycki score) (8) is also reported.

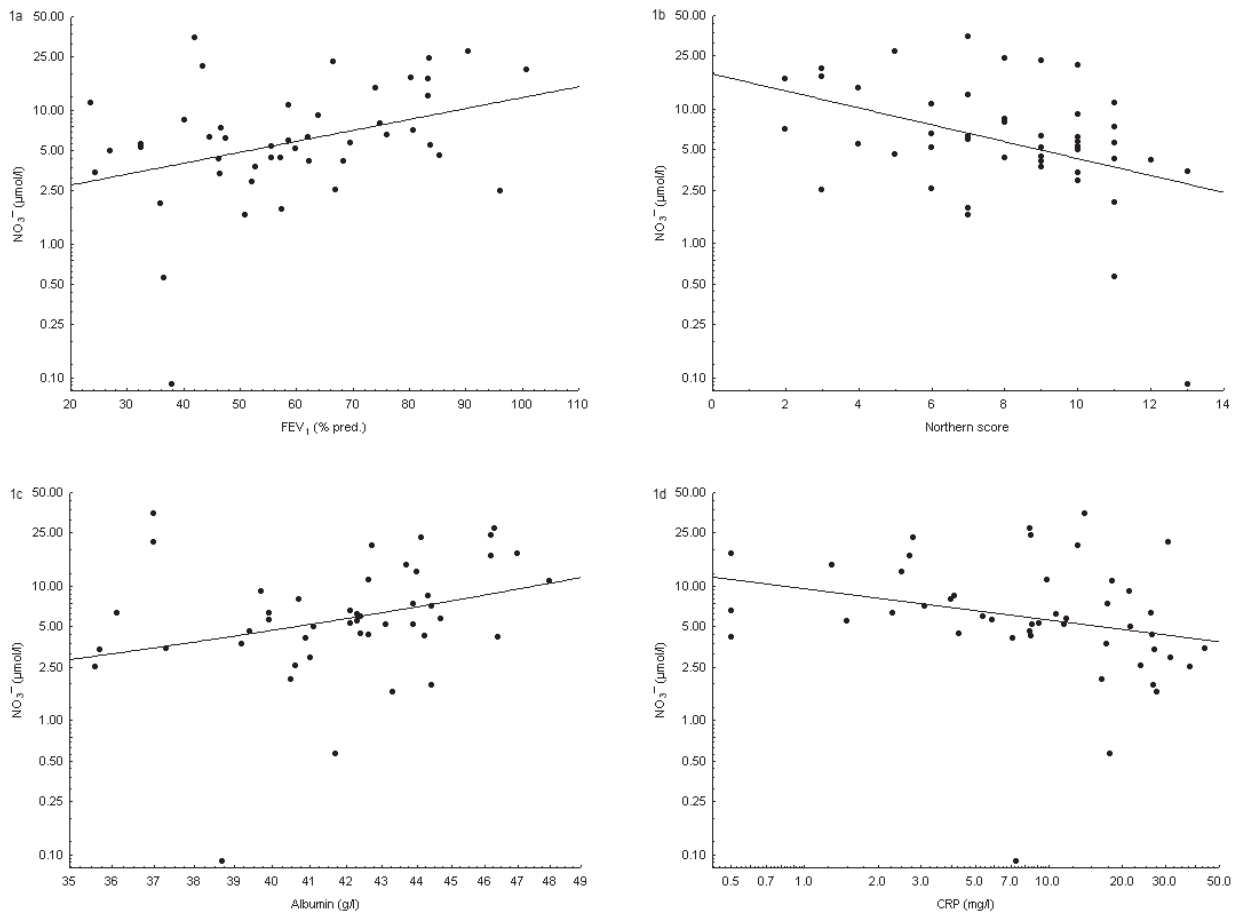
The issues regarding eNO levels in CF seem to be different in children with early lung disease than in adults with advanced bronchopulmonary involvement. The eNO value in children is initially normal and decreases subsequently (19). Moreover, children with CF have a higher alveolar NO concentration together with

normal bronchial NO flux (20). This is confirmable with a higher macrophages count in alveoli in CF fetuses before infection (25) and with increased expression of inducible NO synthase (iNOS) in inflammatory cells (26). The expression of iNOS in airway epithelial cells is normal (9) or decreased (23).

On the other hand, alveolar NO concentration is normal and bronchial NO flux is decreased in CF adults with advanced lung disease (4). The expression of iNOS in both alveolar macrophages and airway epithelial cells decreases as the inflammation progresses and is inversely related to the neutrophils count in bronchoalveolar lavage fluid (9).

Beside iNOS, the levels of eNO are influenced by arginase activity. Arginase reduces the availability of arginine for NO synthase. Arginase activity in CF is higher and negatively correlated to FEV<sub>1</sub>. Antibiotic therapy reduces arginase activity in CF (3). Studies with oral L-arginine supplementation in CF patients were conducted. However, they showed diverse results with regard to eNO levels (27, 28).

A further factor taking part in lowered eNO in CF is the retention of NO in respiratory secretions. It was found that similarly to FEV<sub>1</sub>, eNO undergoes changes after dornase alpha treatment (29). In such condition, NO is subject of oxidative metabolism, which is increased in CF airways (30).



**Fig. 1. Correlations between EBC NO<sub>3</sub><sup>-</sup> concentration and clinical parameters in CF patients. Significant correlations were found between exhaled breath condensate NO<sub>3</sub><sup>-</sup> concentration and FEV<sub>1</sub> (1a), Northern score (1b) and serum albumin (1c) and C-reactive protein (1d) concentration.**

We should also take into consideration the bacterial metabolism of NO in CF airways. In chronic *P. aeruginosa* infection, NO is reduced to ammonia (31).

The influence of alimentary  $\text{NO}_2^-$  and  $\text{NO}_3^-$  on eNO in CF is subject of conjecture. In healthy persons, diets rich in  $\text{NO}_3^-$  lead to an increase in eNO (32). Since 80 % of alimentary  $\text{NO}_3^-$  originates in vegetables (33), we cannot exclude the influence of food on low eNO in CF. The reason is that high-calorie, high-fat diet in CF patients may contain a low amount of vegetables. However, CF patients have higher serum concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  than controls (26).

The issues regarding  $\text{NO}_2^-$  and  $\text{NO}_3^-$  concentrations in EBC are also complex. They include the production of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  by oxidative metabolism of NO in airways together with secretion of alimentary  $\text{NO}_3^-$  in saliva and their reduction to  $\text{NO}_2^-$  by oropharyngeal bacteria (34). Swallowed  $\text{NO}_2^-$  may be further reduced in acid juice in stomach to NO.  $\text{NO}_3^-$ -rich diet leads to higher eNO in healthy people, in whom both alveolar NO concentration and bronchial NO flux increase. Chlorhexidine mouthwash leads to a decrease in alveolar NO concentration at baseline and after dietary  $\text{NO}_3^-$  load (35). In CF patients and in healthy controls, chlorhexidine mouthwash leads to a decrease in eNO and EBC concentration of  $\text{NO}_2^-$ . EBC concentration of  $\text{NO}_2^-$  positively correlates with salivary  $\text{NO}_2^-$  concentration. Furthermore, a decrease in  $\text{NO}_2^-$  concentration in saliva after chlorhexidine mouthwash is coupled with an increase in  $\text{NO}_3^-$  concentration. However, EBC concentration of  $\text{NO}_3^-$  is not affected by oropharyngeal disinfection (15). In accordance with these findings, EBC concentration of  $\text{NO}_2^-$  in CF patients is not correlated to pulmonary function ( $\text{FEV}_1$ ), chest radiograph (Crispin-Norman score) and clinical status (modified Shwachman-Kulczycki score) and also do not predict pulmonary exacerbation (36). In our study, we obtained similar results.

There is a lack of data dealing with the relation of EBC  $\text{NO}_3^-$  concentration and clinical and laboratory parameters in CF. In our study, we found a significant correlation of EBC concentration of  $\text{NO}_3^-$  with pulmonary function, chest radiograph score and serum concentrations of albumin and CRP. On the basis of these results, the measurement of EBC concentration of  $\text{NO}_3^-$  in CF patients seems to give more valuable information than the examination of EBC  $\text{NO}_2^-$  concentration. However, the bacterial metabolism of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  in lower airways (similarly to oropharyngeal tract) could contribute to a decrease in  $\text{NO}_3^-$  concentration in EBC. It was shown that the presence of membrane-bound nitrate reductase is critical for *P. aeruginosa* growth in CF respiratory secretions (37) and that sputum concentration of  $\text{NO}_3^-$  increases after antibiotic treatment of pulmonary exacerbation in CF (7).

Correlations of EBC concentration of  $\text{NO}_3^-$  and serum albumin and CRP may be considered surprising. Serum albumin is one of nutritional markers as well as an important antioxidant. The relation of nutritional status and NO synthase activity was studied in rats. In malnourished rats, NO synthase activity was decreased (38). Similarly, malnourished *CFTR*  $-/-$  mice had a decreased amount of  $\text{NO}_2^-$  in lung tissue (39). Positive correlation of EBC concentration of  $\text{NO}_3^-$  with serum albumin concentration could reflect higher NO production in persons with better nutritional status. However, we found no correlation of EBC  $\text{NO}_3^-$  concentration with BMI.

Serum CRP concentration in our CF population may be considered too high for a stable phase of disease. Nevertheless, our values correspond to data in CF adults in other studies (40, 41). Moreover, an airway colonisation with *P. aeruginosa* and/or *B. cepacia* complex was present in our study group in 87 % of subjects and measurements in such groups of CF adults by others give similar data (42, 43). The information about relations of EBC concentration of both  $\text{NO}_2^-$  and  $\text{NO}_3^-$  with systemic inflammatory markers is sparse. For example, Ho and co-workers found a positive correlation of EBC  $\text{NO}_2^-$  concentration and circulating plasma leukocytes and neutrophils (44). In our study, the negative correlation of EBC  $\text{NO}_3^-$  concentration and serum CRP concentration probably reflects the systemic inflammatory response to the severity of bronchopulmonary involvement or above mentioned airway colonisation with typical gramnegative bacteria (45, 46).

EBC concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in healthy subjects observed in our study were in excellent agreement with the results of other investigators obtained using the same collection device and analytical method (47, 48).

In summary, EBC  $\text{NO}_3^-$  concentrations correlate with disease severity in CF patients and are lower than in healthy subjects.

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