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Oral bacteria — The missing link to ambiguous findings of exhaled nitrogen oxides in cystic fibrosis

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Summary
Background: Nitrite in exhaled breath condensate (EBC) has been shown to be elevated in cystic fibrosis (CF), while exhaled nitric oxide (FENO) is paradoxically low. This has been argued to reflect increased metabolism of NO while its diffusion is obstructed by mucus. However, we wanted to study the possible influence of salivary nitrite and bacterial nitrate reduction on these parameters in CF patients by the intervention of an anti-bacterial mouthwash.
Methods: EBC and saliva were collected from 15 CF patients (10–43 years) and 15 controls (9–44 years) before and 5 min after a 30 s chlorhexidine mouthwash, in parallel with measurements of FENO. Nitrite and nitrate concentrations were measured fluorometrically.
Results: EBC nitrite, but not nitrate, was significantly higher in the CF patients (median 3.6 vs 1.3 μM in controls, $p < 0.05$) and decreased after mouthwash in both groups (3.6–1.4 μM, $p < 0.01$; 1.3–0.5 μM, $p < 0.01$). Salivary nitrite correlated significantly to EBC nitrite ($r = 0.60$, $p < 0.001$) and decreased correspondingly after chlorhexidine, whereas salivary nitrate increased. FENO was lower in CF and the difference between patients and controls was accentuated after mouthwash (5.4 vs 8.4 ppb in controls, $p < 0.05$).
Conclusion: EBC nitrite mainly originates in the pharyngo-oral tract and its increase in CF is possibly explained by a regional change in bacterial activity. The limited lower airway contribution supports the view of a genuinely impaired formation and metabolism of NO in CF, rather than poor diffusion of the molecule.

Introduction

The formation of nitric oxide (NO) is influenced by inflammatory activity in the airway mucosa and measurements of exhaled NO (FENO; fraction of expired NO) can be used for

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evaluating the activity of different inflammatory airway conditions.[1,2] These are, in general, associated with an *increase* of FENO, which is believed to originate from an up-regulation of the inducible nitric oxide synthase (iNOS) in the airway epithelium, as shown in asthmatics.[3–5] However, repeated studies on patients with cystic fibrosis (CF) have shown similar[6,7] or even *decreased*[8,9] levels of FENO as compared to healthy controls. This has been considered a paradox, since CF is characterised as a disorder with an intense airway inflammation, in parallel with often chronic colonisations of bacterial pathogens, and a progressive impairment of lung function.[10] Poor diffusion of NO through the thick mucus of the CF airways, a reduced expression of epithelial iNOS, and substrate deficiency (i.e. lack of L-arginine) have been argued to explain this inconsistency, but the issue is still unclear.[11,12]

Analyses of soluble NO metabolites in exhaled breath condensate (EBC) have been suggested as a simple, yet non-invasive, alternative method to recordings of FENO, which could provide further information on the role of nitrogen oxides in the pathology of airway inflammation.[13] Nitrite and nitrate, for example, can be formed through oxidation of NO and their levels in EBC could possibly reflect iNOS activity. Of these two, nitrite is the most extensively studied in EBC and increased levels have been reported in paediatric and adult asthma,[14,15] as well as in CF.[16,17] EBC nitrate is shown to be elevated in asthmatics and smokers,[18] whereas data are deficient regarding CF. The combination of increased EBC nitrite and low FENO in CF has been argued to reflect a genuinely induced formation of NO, but with its diffusion being impaired and therefore rather metabolised to nitrite.[11,16,17] However, there is also a possible source of nitrite and nitrate through salivary secretion in the upper airway region. A large part of the body's circulating nitrate, from endogenous metabolism or dietary sources, is excreted with the saliva and reduced to nitrite in the pharyngo-oral tract by local bacteria.[19] This salivary nitrite generates NO through further non-enzymatic reduction[20] and can thereby influence measurements of FENO.[21,22] More recently it has also been demonstrated that salivary nitrite contributes to EBC nitrite.[23] Since the formation of salivary nitrite might be affected in CF patients, given their potentially different bacterial environment in upper and lower airways, this contribution could be further influenced.

Therefore, we wanted to analyse nitrite and nitrate in EBC and saliva, in parallel with measurements of FENO, before and after the application of an anti-bacterial mouthwash in children and adults with CF and compare the results to values from age-matched healthy controls.

Methods

Subjects

The 15 patients with CF (age 10–43 years, 7 females) were non-smokers recruited from the CF outpatient clinic in Uppsala University Hospital, Sweden. Twelve were colonized with opportunistic bacterial strains in their airways, according to current sputum cultures; i.e. *Pseudomonas* ($n = 7$), *Staphylococcus aureus* ($n = 5$), *Klebsiella* and *Xanthomonas*. Ten of the colonized subjects were under treatment with oral and/or inhaled antibiotics (e.g. tobramycin). All 15 received regular treatment with bronchodilators and expectorants. Five of the CF patients were treated with inhaled corticosteroids. A few had mild airway symptoms, such as cough, but none had an acute exacerbation upon testing. Patient data were compared with the results obtained from 15 non-smoking healthy controls (age 9–44 years, 9 females) – with no previous history of allergy or airway disease.

The study was approved by the regional ethics committee and all the subjects were included after written informed consent from the subjects or their parents (<18 years).

Measurement of FENO

Measurements of FENO were performed according to the ATS/ERS guidelines[2] with chemiluminescence technique using the Niox® (Aerocrine AB, Solna, Sweden). Exhalation flow rate was set to 0.05 l/s, ensured by the analyser's dynamic resistance – if oral pressure was kept between 8 and 20 cm H_2O. NO concentration (ppb) was registered as the mean value from three 10 s exhalations.

Collection of exhaled breath condensate and analysis of nitrite and nitrate

The EBC was collected in a commercial breath condenser (EcoScreen, Jaeger, Würzburg, Germany) during tidal breathing, while wearing a nose-clip. Total exhalation volumes were monitored to 60 l by a spirometer (SpirPro+, Jaeger, Germany) attached to the condenser, providing about 0.5–1.0 ml of EBC fluid. The Teflon-coated collecting tube of the condenser was then centrifuged for 1 min at 400 g before aliquoting the condensate fluid to sample tubes for −20 °C storage and later analysis.

Random samples were analysed for salivary amylase (EnzCheck Amylase Assay Kit, Molecular Probes Inc, Eugene, OR, USA), to confirm negative direct salivary admixture in the EBC fluid (detection limit 0.1 IU/l, corresponding to 0.3 ng/l protein). All tested samples were negative in this highly sensitive assay. To avoid nitrite/nitrate contamination a plasma polymerisation was deposited on the existing Teflon-coating of the condenser tubes (in-house method at Institute for Surface Chemistry, Stockholm, Sweden) and they were thoroughly cleaned in nitrogen-free disinfectant (Descogen, Jaeger, Germany) between each sampling.

The samples were analysed for nitrite and nitrate with a commercial fluorometric assay including DAN (2,3-diaminonaphthalene) reagent (Cayman Chemical Nitrate/Nitrite Fluorometric Assay Kit, Ann Arbor, MI, USA), measuring nitrite concentrations (detection limit 0.2 μM). For nitrate levels, a second reading of fluorescence, after the addition of nitrate reductase, was subtracted with the initial one. Analysis was performed within two months, during which period nitrite and nitrate maintain stable according to tests in our lab.

Saliva collection

Unstimulated saliva from each subject was collected in a polyethylene tube and centrifuged at 400 g for 2 min. A

supernatant of 200 μl was aliquoted and stored in −20 °C for later analysis of nitrite and nitrate, as described above.

Mouthwash with chlorhexidine

Each of the procedures above was repeated 5 min after a 30 s mouthwash with an anti-bacterial solution (0.2% chlorhexidine gluconate with 0.01% menthol).

Statistical analysis

For group comparisons nonparametric Mann—Whitney test was used. For within-group analysis of paired observations, i.e. before and after chlorhexidine mouthwash, nonparametric Wilcoxon signed rank test was applied. The results are presented as median values in text and figures (horizontal bars in the scatter-plots). Correlations were analysed with nonparametric Spearman rank test. All statistics were conducted with commercial software (Prism4™, GraphPad Software, San Diego, CA, USA).

Results

Nitrite in EBC and saliva

The median level of EBC nitrite was significantly elevated in the patients with CF as compared to controls (3.6 vs 1.3 μM, $p < 0.05$). Mouthwash with chlorhexidine markedly reduced EBC nitrite, both in the patients with CF (3.6—1.4 μM, $p < 0.01$) and in healthy controls (1.3—0.5 μM, $p < 0.01$). The difference in median nitrite levels between the two groups decreased by 60%, from 2.2 to 0.9 μM, but remained statistically significant (1.4 vs 0.5 μM, $p < 0.01$) due to a lower degree of scattering. For further details, see Fig. 1a.

Salivary nitrite was, in parallel, significantly higher in the patients with CF as compared to controls (median 219.6 vs 55.0 μM, $p < 0.01$). Also here, median levels decreased considerably in both groups after mouthwash with chlorhexidine (219.6—63.5 μM, $p < 0.001$ and 55.0—19.8 μM, $p < 0.01$), but the statistical significance of the higher salivary nitrite content in CF remained (63.5 vs 19.8 μM, $p < 0.01$). See Fig. 1b.

In addition, there was a strong correlation between baseline nitrite levels in EBC and saliva ($r = 0.60$, $p < 0.001$; Fig. 1c), which was still present after mouthwash ($r = 0.44$, $p < 0.05$; not shown).

Nitrate in EBC and saliva

In contrast to nitrite, there were no significant differences of EBC nitrate between the CF group and the controls, either before (median 7.8 vs 8.2 μM, $p = 0.80$) or after chlorhexidine (median 8.8 vs 9.3 μM, $p = 0.59$), as seen in Fig. 2a.

The median level of salivary nitrate was higher in the CF group, but the difference was not statistically significant at baseline due to considerable spread of data (median 189.8 vs 31.0 μM, $p = 0.20$). However, after a general increase in salivary nitrate after mouthwash (CF: 189.8—964.3 μM, $p < 0.01$; controls: 31.0—226.8 μM, $p < 0.05$) the nitrate content in CF saliva also proved significantly higher (964.3 vs 226.8 μM, $p < 0.05$). See Fig. 2b.

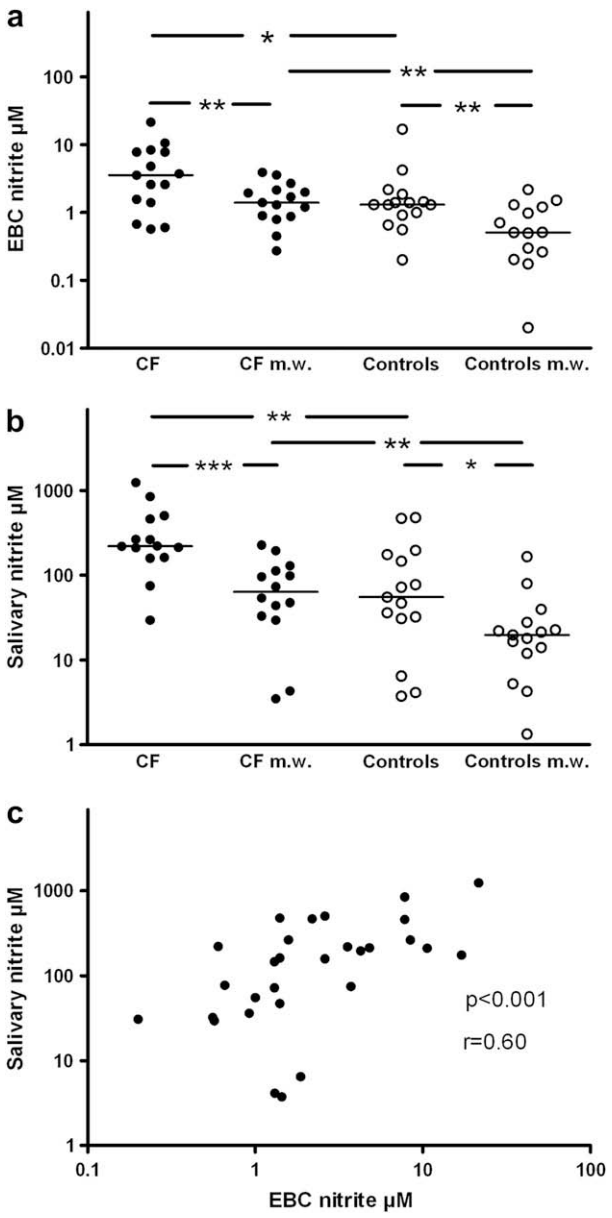

Figure 1 The influence of salivary nitrite, and its generation from bacterial nitrate reductase, on nitrite concentrations in exhaled breath condensate (EBC) from patients with cystic fibrosis ($n = 15$) and controls ($n = 15$). Nitrite was analysed in (a) EBC and (b) saliva before, and 5 min after, a 30 s mouthwash with anti-bacterial chlorhexidine. (c) Demonstrates the correlation between salivary and EBC nitrite at baseline. Note logarithmic concentration scales. CF = cystic fibrosis, m.w. = after mouthwash. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

No significant correlation could be established between nitrate in EBC and saliva ($r = 0.28$, $p = 0.15$; Fig. 2c).

Exhaled nitric oxide

There was a statistical trend towards lower levels of FENO in the patients with CF as compared to controls (median 9.5

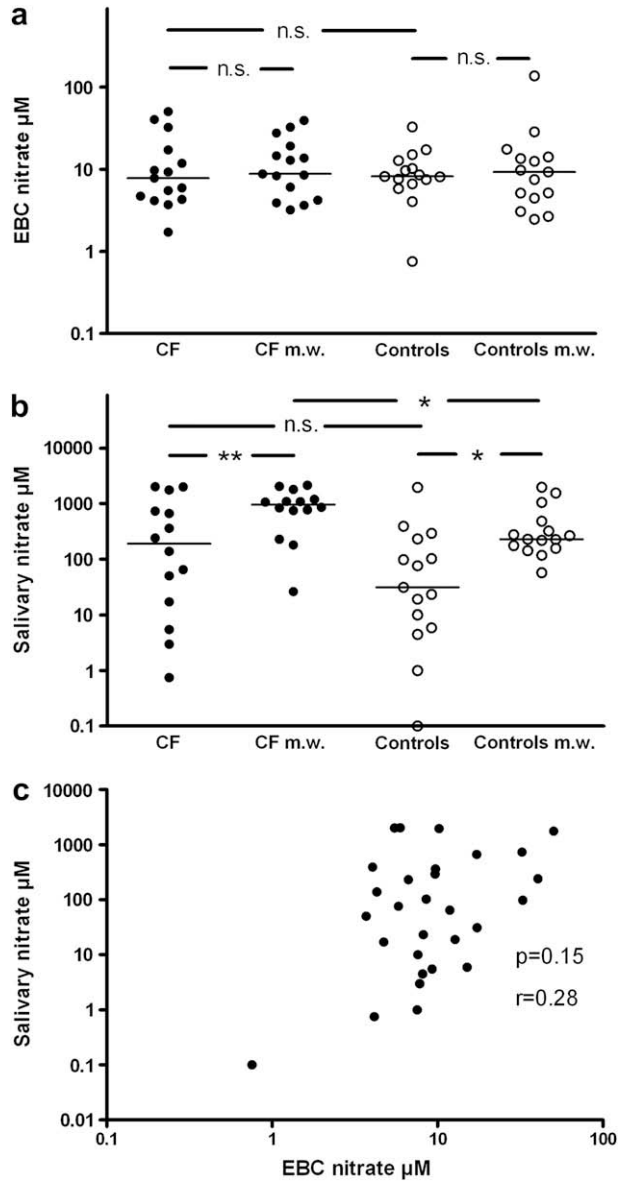

Figure 2 Nitrate concentrations in (a) exhaled breath condensate (EBC) and (b) saliva from cystic fibrosis patients ($n = 15$) and controls ($n = 15$) before and after mouthwash with anti-bacterial chlorhexidine. There was no significant correlation between nitrate concentrations in saliva and EBC as demonstrated in (c). Note logarithmic concentration scales. CF = cystic fibrosis, m.w. = after mouthwash, n.s. = non-significant. $*p < 0.05$, $**p < 0.01$.

vs 10.6 ppb, $p = 0.081$) at baseline. When chlorhexidine was applied, FENO levels not only markedly dropped for both CF patients (median 9.5—5.4 ppb, $p < 0.001$) and controls (10.6—8.4 ppb, $p < 0.0001$), but it also increased the difference in FENO between the two groups, making the lower levels in the CF group statistically significant (5.4 vs 8.4 ppb, $p = 0.023$). See Fig. 3.

Baseline levels of FENO showed no significant correlation to EBC nitrite or nitrate ($r = -0.29$, $p = 0.12$ and $r = 0.19$, $p = 0.31$, respectively).

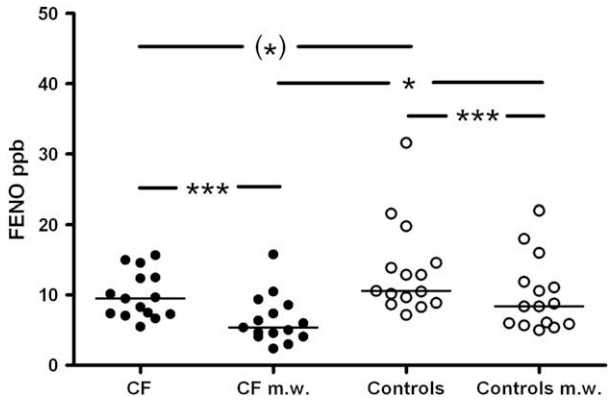

Figure 3 Salivary contribution to exhaled nitric oxide (FENO) in cystic fibrosis patients ($n = 15$) and controls ($n = 15$), as demonstrated by decreased levels after anti-bacterial mouthwash — preventing oral nitrite formation. FENO was measured before, and 5 min after, chlorhexidine application. CF = cystic fibrosis, m.w. = after mouthwash. $(*)p = 0.08$, $*p < 0.05$, $***p < 0.001$.

Discussion

In this study on CF patients we explain the discrepancy of elevated exhaled NO metabolites with simultaneously low levels of FENO by demonstrating an influence from saliva and bacterial activity in the oro-pharyngeal tract.

The median level of EBC nitrite was markedly higher in the CF group and differed significantly from the controls, which compares well with previous studies.[16,17] However, chlorhexidine mouthwash, which has a very potent anti-microbial effect,[24] reduced the EBC nitrite levels by more than 60%, in both the CF and control group, and decreased interindividual variation. Since there is a substantial amount of nitrite in the saliva, which is formed through conversion of salivary nitrate by the bacterial enzyme nitrate reductase,[19] these findings imply that the major part of EBC nitrite originates in the pharyngo-oral tract. Nitrate reductase is predominantly found in facultative anaerobics on the posterior surface of the tongue[25] and its reducing activity can be effectively blocked by chlorhexidine mouthwash.[26] In fact, the presence of nitrite in the saliva is completely dependent upon bacterial reduction of nitrate, which is demonstrated by a total lack of nitrite in saliva collected directly from the parotid glands[27] and in saliva from germ-free rats.[20] The bacterial formation of nitrite represents a symbiotic relationship which provides host defence against microbial pathogens in the oral cavity and the gut through the further non-enzymatic reduction of nitrite to NO.[20] The comparatively higher levels of nitrite in both EBC and saliva from CF patients indicate that they have an altered activity of nitrate reducing bacteria, perhaps even of bacteria in general, in the oral cavity.

The pharyngo-oral origin of EBC nitrite is further supported by the strong correlation observed between the concentrations of nitrite in EBC and saliva in this study. Thus, salivary nitrite was also significantly higher in the CF group than in the controls. After mouthwash, it decreased by approximately 70% in both groups, corresponding well with

the parallel reduction of EBC nitrite. Our data of elevated salivary nitrite in CF are in line with a previous study by Grasemann et al., even though they reported on the joint concentration of salivary nitrite and nitrate and interpreted this as a sign of increased NO activity in the lower airways.[28]

Since the levels of EBC nitrite remained higher in the CF group even after the anti-bacterial mouthwash one can speculate whether there is yet another mechanism involved. However, there was a marked reduction by 60% of the difference and such an additional source would then be of secondary importance in terms of nitrite quantities. Furthermore, since also *salivary* nitrite levels remained higher in the CF patients after chlorhexidine the remaining difference in EBC nitrite is more likely explained by a limited ability of the mouthwash to erase all bacterial activity in the oral cavity and pharynx. This could be due to an incomplete antimicrobial effect of chlorhexidine and to the difficulty in rinsing the entire pharyngo-oral tract, as it elicits an unpleasant swallowing reflex. Nevertheless, a minor contribution from nitrate reducing bacteria below the pharynx, which could be more pronounced in the CF airways, is still possible.

In contrast to nitrite, there was no significant increase of nitrate in EBC from the CF patients. With the observed decrease of nitrite after the mouthwash, one could perhaps expect the nitrate concentrations to elevate. This was true for the salivary concentrations of nitrate, but not for EBC nitrate. In addition, there was no correlation between baseline concentrations of nitrate in saliva and EBC, as in the case of nitrite. The most probable explanation for this is that nitrate in EBC mainly originates in the lower airways. However, a quantitatively smaller salivary contribution of nitrate to EBC certainly occurs, but it is likely to be overshadowed by the bronchially derived nitrate. This is supported by a study from Marteus et al. on tracheostomised patients, which showed similar amounts of nitrate in EBC, when collected both through the tracheostomy and through oral breathing, whereas EBC nitrite was almost exclusively found in the orally exhaled air.[23]

Nevertheless, since the CF group presented an over-all elevated content of salivary nitrate, but no increase at all of EBC nitrate, one could assume that their proportion of bronchial nitrate is lower than in the control group. This corresponds well with the low levels of exhaled NO in CF and recent studies which demonstrate that induction of NO synthesis in the airways generates formation of nitrate, rather than nitrite.[29] Consequently, an increase of EBC nitrite but not nitrate, as in this study, does hardly reflect an induced bronchial NO production, but rather suggests an alternative source of the nitrite — i.e. the salivary one.

The CF patients presented low levels of exhaled NO, which is consistent with previous findings.[11] However, given the intense inflammatory activity in the CF airways, this moderate NO output is puzzling and not yet fully explained. One contributing factor could be that the inflammatory process in CF is characterised by a predominantly neutrophilic activity,[10] whereas iNOS expression and exhaled NO mainly increase by eosinophilic inflammation.[30] That argument is not fully convincing, though, as even viral respiratory tract infections in non-CF patients present increased levels of FENO.[31,32] Another often suggested explanation is attributed to the thick and characteristic mucus that lines the airway epithelium in CF and possibly prevents the diffusion of NO to the lumen.[11] This has been proposed to cause an accumulation of nitrogen metabolites, as shown in airway samples from CF patients, and argued to disguise a genuinely increased NO formation. Apart from elevated nitrite in EBC[16,17] and increased nitrite/nitrate in saliva,[28] there are CF studies demonstrating elevated nitrotyrosine in EBC[33] and lung tissue,[34] as well as raised content of nitrotyrosine and nitrate in sputum.[35] However, this obviously conflicts with our data as we cannot show increased formation of nitrite and nitrate in the lower airways of CF. The reported increase of nitrotyrosine in CF could possibly be explained by an elevated presence of superoxide, due to a more active neutrophilic inflammation, rather than an increased NO metabolism. Elevated sputum nitrate could, furthermore, consist of a salivary contribution or be a result of decreased water content in CF sputum[36] and hence a general increase in the concentration of just about any substance analysed. Therefore, it is more likely that the low levels of FENO in CF are caused by a genuinely altered formation of NO. Findings of a decreased expression of iNOS in the CF bronchial epithelium,[37,38] as well as an impaired signalling system *in vitro* of this enzyme,[39] constitute strong support for this. The markedly lower nasal NO levels reported in CF patients,[6,40] along with reduced expressions of NOS-enzymes in their nasal mucosa,[41] indicate the same. Furthermore, in performed studies on FENO in CF, the bronchially derived proportion is probably overestimated, as we could demonstrate an increased contribution to exhaled NO from the saliva and the non-enzymatic reduction of nitrite with the chlorhexidine mouthwash. Since NO is believed to have beneficial properties in the inflamed airway mucosa, such as bronchodilatation and host–defence against microorganisms, the reduced presence of NO might contribute to the pathology of the CF lung.[11]

Finally, regarding the chlorhexidine mouthwash, we vindicate that its effects on nitrite in EBC and saliva, and consequently on FENO, are explained by diminished bacterial conversion of nitrate to nitrite in the oral cavity and pharynx. Chlorhexidine has sustained bacteriostatic and bactericidal properties[24] and it has shown to be particularly efficient in suppressing the activity of nitrate reductase.[26] The anti-bacterial effect of mouthwash and dental topical application with chlorhexidine is well documented and bacterial growth has been shown to be markedly reduced in saliva, sputum and tissue cultures taken after its administration.[42–44] It could perhaps be argued that the reduced content of nitrite in EBC and saliva after chlorhexidine is more of a rinsing effect, but then it would not cause an increase in salivary nitrate, as we observed. Furthermore, previous studies have not shown any effect on either nitrite in EBC and saliva[23] or FENO[21] from rinsing the mouth with plain water.

Therefore, we conclude that elevated levels of EBC nitrite in CF patients originate in the saliva, through increased activity of pharyngo-oral bacteria, and are not the result of an induced NO formation in the bronchi and lower airways. Together with the moderate levels of EBC nitrate and the results of FENO this supports the idea of a genuinely restrained NO synthesis in the respiratory epithelium of CF, as there are no further traces of increased

NO metabolism in spite of the pronounced chronic infection. Thus, the paradox of low exhaled NO from the inflamed airways of CF is not primarily explained by its trapping behind a mucus barrier but rather by an impaired function or expression of the NO producing enzymes.

Conflict of interest

Wilhelm Zetterquist — no conflict of interest
Helena Marteus — no conflict of interest
Pia Kalm-Stephens — no conflict of interest
Elisabeth Näs — no conflict of interest
Lennart Nordvall — no conflict of interest
Marie Johannesson — no conflict of interest
Kjell Alving — is an associate of Aerocrine AB (manufacturer of NO-analysers)

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