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Single breath N_2-test and exhaled nitric oxide in men

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Summary The N_2 slope is an index of inhomogeneous distribution of ventilation and has been suggested to be suited for early testing of chronic obstructive pulmonary disease (COPD) in smokers. The aim of the present study was to examine the association between the fraction of exhaled nitric oxide (FENO) and the N_2 slope in a random population of smoking and non-smoking men. Altogether 57 subjects were included in the study, 24 never-smokers, seven ex-smokers and 26 current smokers. Subjects were examined twice, in 1995 when they regarded themselves as healthy, and in a follow-up in 2001. Spirometry, N_2 slope and high-resolution computed tomography (HRCT) were performed in 1995 while the follow-up examination included also measurement of FENO.

The FENO value was significantly lower and the N_2 slope higher in current smokers. In smokers but not in never- or ex-smokers FENO was correlated to the difference in N_2 slope between 1995 and 2001 ($r_s = 0.49$, $P = 0.01$). We analysed the data by multiple linear regression adjusted for smoking, mild respiratory symptoms and inhaled steroids. There were significant associations between FENO and the N_2 slope both in 1995 and in 2001. The strongest association was found to exist with the change in N_2 slope during these years.

Sixteen of the subjects could be classified as having COPD, six with mild and ten with moderate COPD. There was a trend for an increase in N_2 slope with increased severity of COPD; among subjects with no COPD the N_2 slope in 2001 was 2.3% N_2/L, and those with mild and moderate COPD had 2.5% N_2/L and 3.9% N_2/L, respectively ($P = 0.0004$). No such trend was seen for FENO (17.8, 15.5 and 20.3 parts per billion (ppb), respectively, $P = 0.8$).

The results show that FENO is associated with the N_2 slope, indicating that FENO reflects inflammatory changes in the peripheral airways of both non-smoking and smoking subjects.

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Background

There is increasing evidence to suggest that the fraction of exhaled nitric oxide (FENO) can be used as a marker for airway inflammation, at least in allergic asthma. Accordingly FENO has so far been used mainly in studies of asthma, including a few studies exploiting its ability to predict exacerbations of asthma.[1,2] In studies of chronic obstructive pulmonary disease (COPD) the usefulness of FENO is more ambiguous[3] while smoking has been reported to lower FENO.[4,5] A number of studies[6–8] have shown that patients with COPD have a lower FENO than do healthy individuals and that smoking status highly influences FENO levels. Among ex-smoking subjects; however, those with COPD have higher FENO levels than those without COPD.[9] The FENO value has also been shown to increase during exacerbations of COPD[9,10] and to be correlated to neutrophils in induced sputum among patients with COPD,[11] indicating that the method potentially has value in monitoring COPD, at least in non-smoking subjects.

Smokers have a lower FENO than do non-smokers.[5] Directly after smoking a cigarette there is, however, an increase in FENO lasting for at least 10 min[12] and probably due to the high contents of nitric oxide (NO) in inhaled smoke. Smoking cessation is associated with an increase in FENO, though FENO levels have been found to be lower among ex-smokers than among never-smokers still 8 weeks after quitting smoking.[13] Experimental studies have shown that it is mainly endothelial NO synthase (eNOS) that is irreversibly inhibited by cigarette smoke.[14,15]

The slope of the single-breath N_2 test (the N_2 slope) is an index of inhomogeneous or asynchronous intra-pulmonary ventilation distribution.[16–18] The N_2 slope has been shown to be more sensitive than spirometric variables for detecting tobacco smoke-induced changes in the lungs.[19,20] In studies relating structural changes to lung function tests among smokers it has been found that the N_2 slope is related to inflammatory changes in the peripheral airways.[21] Furthermore, the N_2 slope has been shown to be predictive of the decline of forced expiratory volume in 1 s (FEV_1) in smokers.[22] The predictive value of the N_2 slope used in combination with spirometry has also been shown by Stanescu et al. (1998).[23] There is therefore increasing evidence suggesting that the N_2 slope, in particular in combination with spirometry, may be useful as an early test for COPD.

As FENO is a marker of airway inflammation, we hypothesised that it may be an early marker in COPD when smoking habit is adjusted for. In the present study we have therefore examined the relation between FENO levels and the N_2 slope in a population sample of 57 men born in 1933 in Gothenburg, Sweden.

Materials and methods

Study population

The subjects were recruited from an earlier study on a random population sample titled, ''Men born in 1933 in Göteborg''.[24] From the original cohort of 879 men a random sample of 92 men (58 current smokers and 34 non-smokers) were investigated in 1995 concerning emphysematous lesions[25] and inflammatory markers.[26] The FENO value was, however, not obtained at that investigation. At the time the subjects all regarded themselves as healthy and did not report previous medical consultations for respiratory symptoms.

The present study is a follow-up study of those 92 men who were investigated in 1995. Altogether 67 subjects attended the follow-up study, giving a participation rate of 73%. Ten subjects were excluded because of missing NO values (seven due to equipment failure and three due to failure to perform acceptable NO measurements). One of the excluded subjects also failed to perform the N_2 test. Hence, the final number of subjects included in the present study was 57.

Subjects were divided into current smokers, ex-smokers and never-smokers at follow-up. Current smokers included also those three subjects who had stopped smoking during the previous 12 months. The current smokers had smoked for 51 years on average while the ex-smokers had smoked for an average of 37 years. Basic data on the subjects are presented in Table 1.

Examination

Exhaled NO was measured using the Ecophysics Breath Analyzer CLD 700 AL chemiluminescence analyser (Switzerland). A special computer biofeedback system was used to regulate exhalation flow rate (Exhalation Breath Analyzer™; Aerocrine AB, Stockholm, Sweden). Two-point calibration of the analyser was performed daily with a certified NO calibration gas. The exhaled NO was measured during a slow single exhalation for 20 s against an oral pressure of 5 cm H_2O at a constant flow rate of 50 ml/s ($\pm$10%) in accordance with American Thoracic Society (ATS) recommendations.[27] The measurements were performed in triplicate on

Table 1 Mean (± standard deviation, SD) subject data for 2001.

	Never-smokers	Ex-smokers	Current smokers
n	24	7	26
BMI (kg/m^2)	27.0 (2.7)	28.0 (2.0)	25.4 (3.4)
FEV_1 (%)	103 (14)	91 (14)	88 (21)
FVC (%)	105 (15)	99 (11)	100 (18)
D_LCO (%)	98 (14)	99 (19)	79 (18)
Mild respiratory symptoms[a]	6 (25%)	3 (43%)	15 (58%)
Asthma	1 (4%)	0	1 (4%)

D_LCO = diffusing capacity of the lung for carbon monoxide; FEV_1 = forced expiratory volume in 1 s; FVC = forced vital capacity. FEV_1, FVC and D_LCO are expressed as a percentage of the predicted normal values.
[a]Current wheeze, cough or dyspnoea.

each occasion and the mean concentration, in parts per billion (ppb), during the plateau phase was registered.

The subjects underwent spirometry with a water-sealed spirometer, according to European Respiratory Society (ERS) guidelines.[28] Vital capacity (VC) was measured and FEV_1 was registered and its percentage of the VC (FEV%) obtained. The diffusing capacity of the lung for carbon monoxide (D_LCO) was assessed by the single-breath method using standard equipment (Sensor Medics 2200, Sensor Medics Corporation, The Netherlands). The slope of the alveolar plateau (i.e. the N_2 slope) was obtained by the single-breath N_2 method.[29] In the following the results from the measurements in 1995 and 2001 are presented, as is the difference in N_2 slope (i.e. $N_2^{2001} - N_2^{1995}$). Predicted normal values for VC, FEV_1 and FEV% were calculated according to a local reference material,[30] while D_LCO was measured according to Salorinne[31] and for the N_2 slope according to Sixt et al.[32]

High-resolution computed tomography (HRCT) was used and evaluated as previously described[33] in all subjects. In brief, the diagnosis of emphysematous lesions was based on the findings of areas with low attenuation and/or presence of stretched narrow vessels. Severity of emphysematous changes was scored on a four-point scale ranging from 0 to 3, where 0 was equivalent to no emphysema. Ground-glass attenuation was defined as slightly hyperattenuating areas in which underlying vessels and bronchial walls remained visible. All subjects completed a respiratory questionnaire with items similar to those of previous questionnaires.[34]

Statistical methods

The statistical analyses of the categorical data were based on the χ^2 test. As the NO data were not normally distributed they were analysed with non-parametric methods (Kruskal-Wallis), but group means are presented. *P*-values have usually been provided. Correlations were tested using Spearman's rank correlation coefficient. The associations between the log-transformed values of exhaled NO and different explanatory variables (smoking, height, use of inhaled glucocorticoids, asthma symptoms and N_2 slope at follow-up) were tested using multiple linear regression models. The significance of the slope in the multivariate regression model was based on the *t* distribution. For the linear regression models, PROC REG in the SAS statistical package (release 8.0; SAS Instituted, Cary, NC, USA) was used.

Results

The FENO value in relation to smoking status is presented in Fig. 1. The FENO values were significantly lower among current smokers than in never-smokers and tended to be higher among ex-smokers ($n = 7$) than among the never-smoking subjects but the difference was not statistically significant.

FENO, N_2 slope for 1995 and 2001, and the change in N_2 slope are presented in Table 2. FENO was significantly lower and the N_2 slope higher among the current smokers. Among the never-smokers and ex-smokers there was no association between FENO and the N_2 slope in 1995 or 2001; nor was there an association between the FENO value and the change in N_2 slope (Fig. 2a). Among the current smokers there was a significant association between FENO values and the change in N_2 slope from 1995 to 2001 ($r_s = 0.49$, $P = 0.01$) (Fig. 2b), but not between FENO and the single N_2 slope in either 1995 or 2001. When analysing the data by multiple linear regression adjusted for smoking, height, mild respiratory symptoms and inhaled

steroids, there were significant associations between FENO values and the N_2 slope for 2001 (estimate 0.13, $P = 0.051$). The strongest association was found with the change in N_2 slope (see Table 3). The latter model included the result for the N_2 slope in 1995, thereby weighting the change in N_2 slope to the initial value.

The seven ex-smokers had the highest FENO levels (24.1 ppb) and the largest change in N_2 slope from 1995 to 2001 (1.39% N_2/L), compared with never-smokers (19.0 ppb and 0.28% N_2/L) and smokers (15.4 ppb and 0.45% N_2/L).

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria,[35] 16 of the subjects could be classified as having COPD, six (one never-smoker, one ex-smoker and four current smokers) with mild and ten (one ex-smoker and nine current smokers) with moderate COPD. There was a trend for an increasing N_2 slope with increasing severity of COPD; among subjects with no COPD the N_2 slope in 2001 was 2.3% N_2/L; those with mild and moderate COPD had 2.5% N_2/L and 3.9% N_2/L, respectively ($P = 0.0004$). There was no such trend for FENO (17.8, 15.5 and 20.3 ppb, respectively, $P = 0.8$).

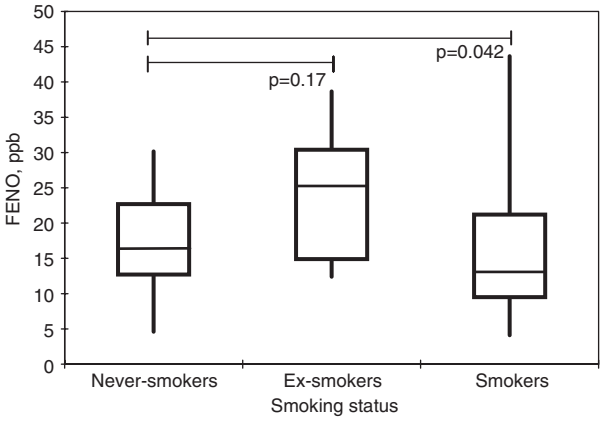


Figure 1 Exhaled nitric oxide (NO), (ppb), in relation to smoking status, in box-plots representing maximum and minimum values, 25th and 75th percentiles and medians.

There was no association between FENO and FEV_1, or FENO and change in FEV_1 from 1995 to 2001, nor did analyses of the data for the group as a whole or analyses stratified according to smoking status reveal any associations. Similarly, HRCT showed no association between FENO and emphysema, or progress of emphysematous lesions, between 1996 and 2001.

Six non-smokers (three never-smokers and three ex-smokers) were classified as having ground-glass attenuation, as demonstrated by HRCT. In this group neither the N_2 slope nor the change in N_2 slope was different from those without ground-glass attenuation (0.52% vs. 0.56% N_2/L, $P = 0.8$). There was also no significant difference in FENO value between the groups (18.0 vs. 20.7 ppb, $P = 0.33$) or in FEV_1 or FVC. In the small group of subjects with ground-glass attenuation the correlation between the change in N_2 slope from 1995 to 2001 and exhaled NO measured in 2001 was, however, stronger ($r_s = 0.89$, $P = 0.02$).

Discussion

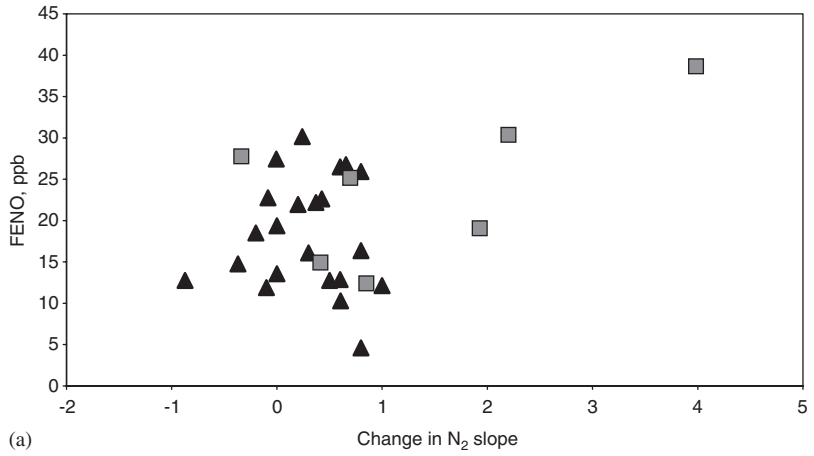
The main result of the present study is that an association was found to exist between FENO and deterioration of the N_2 slope over a 6-year period in a group of never-, ex- and current smokers when adjusting for smoking status. In a multiple regression model, FENO was associated with the deterioration of the N_2 test over six years while the results from the last N_2 test in 2001 were not significantly associated with FENO. The reason for this is unclear but one plausible explanation may be that the deterioration of the N_2 slope is a stronger indicator of an ongoing inflammatory process than is the single last measurement of the N_2 slope.

The FENO value for ex-smokers, 24.1 ppb (range 12.4–38.6), was slightly but not significantly higher than that for the never-smokers, 19.0 ppb (4.6–39.5), indicating that the suppressive effect of previous smoking on FENO had ceased. In the

Table 2 Results of the N_2 test for 1995 and 2001, difference between the two test results, and exhaled nitric oxide (NO), according to smoking habit (means and inter-quartile range).

	Never-smokers	Ex-smokers	Current smokers
N	24	7	26
N_2 test 1995[a]	1.4 (1.0–1.7)	1.7 (0.8–2.1)	2.7 (1.8–3.7)
N_2 test 2001[a]	1.7 (1.4–2.4)	3.1 (1.6–4.3)	3.2 (2.5–3.7)
Difference in N_2 test (2001–1995)[a]	0.3 (0–0.6)	1.4 (0.4–2.2)	0.5 (0.1–1.0)
Exhaled NO 2001 (ppb)	19.0 (12.8–24.4)	24.1 (14.9–30.4)	15.4 (9.8–20.3)

ppb = parts per billion.
[a]% N_2/ L.



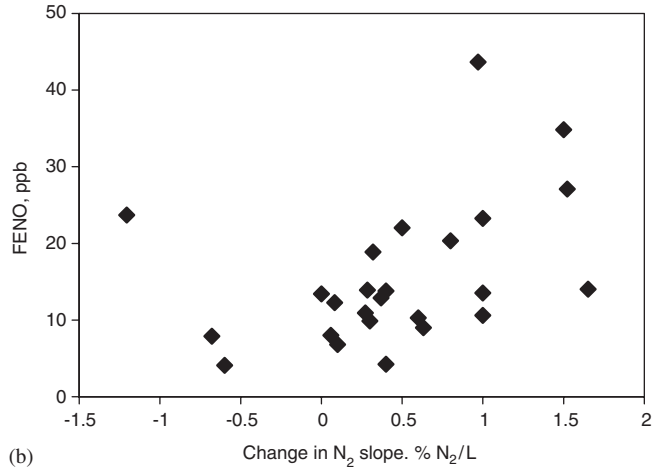


Figure 2 (a) Fraction of exhaled nitric oxide (FENO), in parts per billion (ppb), and change in the N_2 slope between 1995 and 2001 among never-smokers (▲) and ex-smokers (■). Spearman's rank correlation coefficient for never-smokers $r_s = -0.06$, $P = 0.77$, and for ex-smokers $r_s = 0.46$, $P = 0.23$. (b) Fraction of exhaled nitric oxide (FENO), in parts per billion (ppb), and change in N_2 slope between 1995 and 2001 among current smokers. Spearman's rank correlation coefficient $r_s = 0.49$, $P = 0.01$.

Table 3 Variables included in a multiple linear regression model, with logarithm- transformed fraction of exhaled nitric oxide (FENO) as the dependent variable.

Variable	Estimate	SEM	*P*-value
Intercept	2.67	0.16	0.6
Smoking			
Never-smoker[a]	−0.05	0.24	0.8
Current smoker[a]	−0.41	0.24	0.09
Height (cm)	0.02	0.01	0.07
Asthma-like symptoms[a]	−0.15	0.14	0.3
Current use of inhaled steroids[a]	0.42	0.53	0.4
N_2 test, 1995[b]	0.15	0.085	0.08
Difference between N_2 test results of 1995 and 2001[b]	0.22	0.098	0.03

SEM = standard error of the mean.
[a]Yes = 1, no = 0.
[b]% N_2/L.

analysis we also included two subjects who reported treatment with inhaled glucocorticoids. Their FENO values were 30.3 and 39.5 ppb, respectively.

In the literature there is only one previous study on FENO and N_2 slope.[36] In that study FENO was reported to be related to the N_2 slope ($r_s = 0.54$, $P = 0.032$) in a cross-sectional study of 16 subjects with intermittent or mild asthma. The authors concluded that FENO was reflecting small airway dysfunction in asthma and may be complementary to spirometry to monitor asthma.

The N_2 slope is suggested to reflect an inhomogeneous distribution of ventilation, which may result from either inflammatory processes or structural changes in the airways. Inhomogeneous ventilation may also affect the FENO levels per se, since in segments of the lung with low intrabronchial flow the air has more time to be saturated with NO, leading to increased exhaled NO concentrations. On the other hand, to reach the predicted exhalation flow the airflow in other parts of the bronchial tree must be higher, causing a lower concentration of NO in these segments. The net result on the exhaled NO concentration is unclear.

Ground-glass attenuation is related to various patho-anatomical changes in the airways of smokers, such as partial filling of air spaces, inflammatory or fibrotic interstitial thickening or increased capillary blood volume. In the few subjects with ground-glass attenuation in this study the N_2 slope was not increased nor the FENO levels and they had normal spirometry. On the other hand, in subjects who had ground-glass attenuation the association between FENO and N_2 slope was strong ($r_s = 0.89$, $P = 0.02$).

An analysis of the drop-outs in the follow-up study has been performed. Among the subjects who were never-smokers in 1995, the participating and non-participating subjects were quite similar. Among the current smokers the non-participating subjects had lower FEV_1 and FVC compared with the participating subjects. However, the N_2 slope of the non-participating subjects was 2.57, as compared with 2.97 for the participating subjects ($P = 0.43$). Consequently we do not consider the selection bias to have influenced the results to any significant degree.

Whether a rise in FENO is beneficial or has detrimental effects in ex-smokers, such as in four out of seven ex-smokers in this study, is unclear. In other diseases increasing evidence has been presented indicating favourable effects of increased NO production. An important function appears to be that NO has the potential to act as a scavenger and bind more aggressive free radicals. In COPD, in which there is an excess of free radicals, such a scavenger would fulfil an important function. It is, however, uncertain to what extent FENO reflects the actual production of NO in the micro-environment, as the locally produced NO may be largely scavenged by neutrophil-derived radicals.

In conclusion, the results indicate that measurement of FENO can reflect inflammatory changes in the peripheral airways of non-smokers, ex-smokers and current smokers. The clinical implication of this is still unclear. Whether FENO can add valuable information to the slope of the N_2 test, enabling a better prediction of COPD, is of interest for future studies.

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