

Original article

Determinants of increased exhaled nitric oxide in patients with suspected asthma

Exhaled nitric oxide (FE_{NO}) has been proposed as a marker of asthmatic inflammation, but it is unclear whether FE_{NO} in clinical use selects patients primarily according to their atopic or asthmatic status. The aim of this study was to investigate the determinants of increased FE_{NO} in patients with suspected asthma, by means of multinomial logistic regression analysis. The FE_{NO} of 132 patients referred because of symptoms suggestive of asthma were studied, and the explanatory factors tested included atopy according to prick skin tests, clinical asthma according to lung function tests, sputum eosinophilia and bronchial hyperresponsiveness (BHR). Slightly elevated FE_{NO} levels were significantly explained only by sputum eosinophilia (OR: 3.7; 95% CI: 1.1–13.1; *P* = 0.04), but for high levels of FE_{NO} (≥ 3 SD of predicted), clinical asthma (OR: 16.3; 95% CI: 5.4–49.7; *P* < 0.0001) and sputum eosinophilia (OR: 12.0; 95% CI: 4.1–35.0; *P* > 0.0001) were the characteristics with the highest prediction, followed by atopy and BHR. A significant interaction between asthma and atopy was observed relating to the effect on high FE_{NO}, but further analyses stratified by atopy showed significant associations between asthma and high FE_{NO} both in atopic and nonatopic patients. We conclude that in patients with symptoms suggesting asthma, slightly elevated and high levels of FE_{NO} are associated with sputum eosinophilia, whereas asthma is significantly associated only with high levels of FE_{NO}, irrespective of atopy. The results suggest that FE_{NO} is primarily a marker of airway eosinophilia, and that only high values of FE_{NO} may be useful to identify patients with atopic or nonatopic asthma.

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Endogenous nitric oxide (NO) is synthesized from L-arginine by isoenzymes of NO synthetase, some of which are inducible by proinflammatory cytokines. In the exhaled air, NO is detectable in small amounts in healthy individuals, but the fractional concentration (exhaled nitric oxide, FE_{NO}) has been shown to increase in patients with inflammatory disorders of the airways, such as bronchiectasis and lower respiratory tract infections, and especially in asthma (1). Therefore, FE_{NO} has been proposed as a noninvasive marker of airway inflammation in asthma. Accordingly, FE_{NO} has been shown to identify asthmatic adults (2) and children (3) from healthy subjects with high sensitivity and specificity.

Recent studies have shown the relationship between FE_{NO} and markers of mucosal eosinophilic inflammation in patients with asthma (4, 5), although the precise mechanism how these different inflammatory markers are related is not known. Particularly, FE_{NO} seems to be increased in atopic asthmatics, and increased levels of FE_{NO} have been reported also in patients with atopic

rhinitis without asthma (6), atopic asthmatics in remission (4), and in atopic subjects without any atopic disease or asthma (7). Furthermore, Gratzou et al. (8) could not find any difference in FE_{NO} between nonatopic asthmatics and healthy controls, suggesting that it is rather the atopic status than asthma, which is mainly responsible for the higher NO production in the lower airways. These findings are somewhat contradictory, since mucosal eosinophilic inflammation is likely to be present also in nonatopic asthma (9–13).

We wanted to address the question whether FE_{NO} can be used clinically to identify asthma and airway inflammation in a series of patients undergoing diagnostic procedures because of symptoms suggesting asthma (wheeze, cough, dyspnea). We reasoned that these patients represent the most probable target for the clinical use of FE_{NO}. Logistic regression analysis was used to identify determinants for raised FE_{NO} such as clinical asthma, atopy, sputum eosinophilia, and bronchial hyperresponsiveness (BHR). Particularly, the role of FE_{NO} in atopic and nonatopic asthma was of interest.

Material and methods

We included 132 patients remitted to an asthma and allergy clinic because of symptoms suggesting asthma (wheeze, cough, or dyspnea). Their characteristics have been presented in Table 1. The patients were included consecutively provided that satisfactory FE_{NO} measurements and induced sputum specimen were obtained. Patients using anti-inflammatory therapy within 3 months of the study (inhaled or systemic corticosteroids, chromones or leukotriene antagonists), were excluded. At the time of testing, the patients were in a stable condition and none of the patients had experienced a respiratory tract infection in the preceding 2 weeks. Short-acting β_2 -agonists were withheld for at least 12 h preceding the tests.

The patients underwent a series of lung function and allergy testing, including skin prick tests, flow-volume spirometry, bronchial challenge test, measurement of FE_{NO}, and induced sputum analysis. Standardized questionnaires were used to record the primary lower respiratory symptoms prior to referral, such as wheeze, cough, and shortness of breath. In addition, symptoms of rhinitis and smoking habits were recorded as possible confounding factors for FE_{NO} (6, 8, 14–16). The clinical diagnosis of asthma was based on typical symptoms and on at least 20% diurnal variation in peak expiratory flow (PEF), or on at least 15% improvement in forced expiratory volume in 1 s (FEV₁) or PEF in response to bronchodilators, or on at least 15% exercise-induced decrease in PEF or FEV₁. Skin prick testing was carried out with the following common inhalant allergens: birch, grass, mugwort, cat, dog, cow, horse, *Cladosporium herbarum*, *Dermatophagoides pteronyssinus*, and latex (Soluprick SQ, ALK, Horsholm, Denmark). The patient was regarded as atopic if having one or more positive reactions, determined as a wheal diameter of 3 mm or greater in the presence of expected results in control solutions (histamine dihydrochloride 10 mg/ml as a positive and solvent as a negative control).

Flow-volume spirometry was performed according to European standards (17), and BHR was estimated using a dosimetric histamine challenge test (18). By using the dose–response curve, the provocative dose of inhaled histamine producing a decrease of 15% in FEV₁ (PD15FEV₁) was determined. According to PD15FEV₁, the bronchial hyperreactivity was graded as mild (0.41–1.60 mg), moderate (0.11–0.40 mg), or severe (≤ 0.1 mg) (18). The patients with PD15FEV₁ ≤ 1.6 mg were considered hyperreactive. Fractional FE_{NO} concentration was measured using a chemiluminescence analyzer (CLD 77 AM; Eco Physics, Duernten, Switzerland) connected to a computerized system (Exhaled Breath Analyzer, Aerocrine AB, Stockholm, Sweden), and calibrated with a certified NO calibration gas mixture. The

online single exhalation technique with exhalation rate 100 (± 10) ml/s and positive expiratory mouth pressure of 10–20 mmH₂O was applied, according to recommendations in Ref. (19). The mean FE_{NO} of two to three acceptable end-expiratory plateau measurements was calculated. According to the distribution of FE_{NO} in healthy nonsmoking subjects when using similar technique and exhalation rate, the concentration was coded as normal if < 12 ppb, slightly elevated if 12–15 ppb and high if higher than 15 ppb (20).

Sputum was induced by inhalation of 5 ml of 3% NaCl solution, using an ultrasonic nebulizer (Omron U1, Omron, Germany) for 15 min as previously described (21). Sputum samples were transferred to Petri dishes and examined against a dark surface. The more viscous parts were collected, using forceps, and mixed. Air-dried slides were stained using eosin and methylene blue. A sample was considered to be adequate, and to originate from the lower airways, if it contained macrophages and fewer than 50% of squamous epithelial cells. The success rate for representative sputum induction has been evaluated previously and found to be 89–92% (21, 22). All analyses were conducted unaware of the clinical characteristics of the subject. Cell proportions were assessed semi-quantitatively, on a scale from 0 to 4, as previously described (22). Eosinophilia was graded as 0 (no or occasional eosinophils), 1 (scanty), 2 (moderate), 3 (numerous eosinophils), or 4 (predominance of eosinophils). Approximately, grade 0 corresponds to eosinophils accounting for fewer than 1% of all nonsquamous cells, grade 1 to eosinophils accounting for 1–5%, grade 2 for 5–10%, grade 3 for 10–50% and grade 4 to eosinophils accounting for more than 50% of all nonsquamous cells (21, 22). The patient was defined as having significant sputum eosinophilia when the sputum eosinophil grade of 2 or more was observed.

Statistical calculations

Comparisons between continuous variables were estimated with unpaired *t*-test or Mann–Whitney test, depending on the distribution of the variable. For correlation analyses, Spearman rank correlation was used. The relationship of categorical variables with increased FE_{NO} were analyzed by multinomial logistic regression analysis with fixed model design, and by stepwise design with the forward selection method. The effects were given as odds ratios (OR) with 95% confidence intervals (CI). Statistical analyses were performed using STATVIEW and SPSS software packages (version 11.0 for Windows, Chicago, IL, USA).

Results

Of the 132 patients recruited, 61 were skin prick test-positive (atopic) and, based on the lung function criteria, 30 were found to have clinical asthma. In 21 patients, high levels of FE_{NO} were observed, in 13 patients the concentration was slightly elevated, and in 98 patients the FE_{NO} was regarded as normal. Atopic patients had higher mean concentration of NO in exhaled air (13.6 ppb) than nonatopic patients (9.2 ppb, $P < 0.05$). The mean FE_{NO} was markedly increased (21.5 ppb) in the patients with clinically confirmed asthma, compared with those in whom the diagnosis could not be assessed (8.3 ppb, $P = 0.0006$). Accordingly, in the subgroup of nonatopic patients ($n = 71$), the asthmatics ($n = 11$) showed higher mean FE_{NO} (19.6 ppb), compared with

Table 1. Characteristics of the patients with symptoms suggesting asthma

N	132
Sex (males/females)	34/98
Age (years)	41 (14–81)
Height (cm)	168 (145–188)
Smoker (yes/no)	15/117
Atopy (yes/no)	61/71
Rhinitis (yes/no)	82/50
Duration of symptoms (years)	3.8 (1–20)
FEV ₁ (l)	3.3 (1.8–5.9)
FEV ₁ (% predicted)	95 (69–134)
PD15FEV ₁ (mg), median (range)	1.2 (0.1 to >1.6)
Sputum eosinophils (score)	0.4 (0–3)
FE _{NO} (ppb)	11.3 (2.2–74)

Results are expressed as mean (range).

nonasthmatics (7.4 ppb, $P < 0.0001$). In the whole series of patients, there was a significant correlation between FE_{NO} and the grade of sputum eosinophilia ($r = 0.42$; $P < 0.0001$). Similarly, in nonatopic patients, the relationship was significant ($r = 0.38$; $P < 0.002$).

Age, sex and smoking habits were not significantly related to elevated FE_{NO} . Smokers ($n = 15$) and non-smokers ($n = 117$) showed similar mean levels of FE_{NO} (11.1 vs 13.0 ppb; $P = 0.58$, respectively). About 82 patients reported symptoms of rhinitis. Although the atopic patients with rhinitis ($n = 44$) had higher FE_{NO} compared to the patients with rhinitis but without atopy ($n = 38$) (13.5 vs 7.6 ppb, $P = 0.04$), as a group the patients with and without rhinitis showed similar mean levels of FE_{NO} (10.8 vs 12.1 ppb; $P = 0.55$, respectively). Since smoking habits and symptoms of rhinitis *per se* did not have an independent effect on FE_{NO} in the regression analyses ($P > 0.10$), these factors were omitted from subsequent models and analyses.

According to the questionnaire, 38 patients reported wheeze, 94 cough and 83 shortness of breath as their lower respiratory symptom. Slightly elevated FE_{NO} was not significantly related to any of the reported symptoms. For high FE_{NO} , wheeze was the only symptom which was significantly predictive (Table 2).

The relationship of increased FE_{NO} with possible explanatory findings have been shown in Table 3. Slightly elevated FE_{NO} levels were associated with sputum eosinophilia (OR: 3.7; 95% CI: 1.1–13.1; $P = 0.04$), but not significantly with asthma, atopy, or BHR. Clinical asthma was the characteristic with the highest prediction of high FE_{NO} (OR: 16.3; 95% CI: 5.4–49.7; $P < 0.0001$), followed by sputum eosinophilia (OR: 12.0; 95% CI: 4.1–35.0; $P > 0.0001$). The relationship of atopy and BHR with high FE_{NO} was also significant but weaker than that of asthma and sputum eosinophilia. By introducing the main explanatory variables and their interaction terms in stepwise (forward selection) model, clinical asthma (OR: 16.4; 95% CI: 4.6–58.5; $P < 0.0001$) and sputum eosinophilia (OR: 11.7; 95% CI: 3.2–41.7; $P < 0.0001$) were the factors included in the model with the best fit, whereafter the other variables and terms remained insignificant. The logistic regression analyses showed significant interactions between asthma and atopy ($P = 0.001$) as well as between

Table 3. Relationship of explanatory findings and increased exhaled nitric oxide (FE_{NO} , slightly elevated or high) according to multinomial logistic regression analysis in the patients with symptoms suggesting asthma ($N = 132$)

FE_{NO} level	Finding	OR	95% CI	P-value
Slightly elevated (12–15 ppb)	Asthma	1.2	0.3–6.0	ns
	Atopy	1.2	0.4–4.0	ns
	BHR	0.8	0.2–3.0	ns
	Sputum eosinophilia	3.8	1.1–13.1	0.04
High (>15 ppb)	Asthma	16.3	5.4–49.7	<0.0001
	Atopy	3.6	1.3–10.1	0.01
	BHR	3.6	1.2–11.2	0.03
	Sputum eosinophilia	12.0	4.1–35.0	<0.0001

BHR, bronchial hyperresponsiveness; OR, odds ratio; CI, confidence interval.

sputum eosinophilia and atopy ($P = 0.003$) relating to their effects on high FE_{NO} . However, further analyses stratified by atopy showed significant associations between asthma and high FE_{NO} both in atopic (OR: 13.4; 95% CI: 3.3–54.4; $P = 0.0003$) and nonatopic (OR: 17.7; 95% CI: 2.7–117.6; $P = 0.003$) patients.

Discussion

The results showed that in patients with symptoms suggesting asthma, high levels of NO in exhaled air, but not slightly elevated ones, have a strong relationship to clinical asthma, based on conventional lung function criteria. This association was significant both in atopic and nonatopic patients. When asthma and airway eosinophilia were accounted for, atopy did not have independent effect on FE_{NO} . This finding contrasts with some previous reports which suggest that FE_{NO} is primarily a marker of atopy (6, 8, 23, 24). Differences in the study populations may explain some of the disagreement: previous studies investigated population based samples, whereas in the present study, all the subjects were remitted for diagnostic evaluation because of suspicion of asthma, were symptomatic at the time of testing, and steroid naïve. The sample we investigated represents more closely the population where diagnostic tools, such as the measurement of FE_{NO} , will be applied in clinical practice.

As possible confounding factors for FE_{NO} , smoking habits and symptoms of rhinitis were recorded. Smoking is known to be associated with increased BHR, as well as with increased total cell counts and eosinophils in the sputum (25), but with decreased FE_{NO} levels (14). On the contrary, raised FE_{NO} levels have been observed in patients with rhinitis, even in the absence of asthma (6, 8, 15, 16). In the present study, however, the effects of smoking habits and rhinitis were negligible, and they could be omitted from the regression models between FE_{NO} and the explanatory variables.

In the present study, the data was categorized in order to estimate the association between FE_{NO} and the explanatory variables. For FE_{NO} , instead of using a simple

Table 2. Relationship of respiratory symptoms with increased exhaled nitric oxide (FE_{NO} , slightly elevated or high) according to multinomial logistic regression analysis in the patients with symptoms suggesting asthma ($N = 132$)

FE_{NO} level	Symptom	OR	95% CI	P-value
Slightly elevated (12–15 ppb)	Wheeze	2.3	0.7–7.7	ns
	Dyspnea	1.4	0.4–4.7	ns
	Cough	0.8	0.2–3.0	ns
High (>15 ppb)	Wheeze	4.9	1.8–13.1	0.002
	Dyspnea	1.0	0.4–2.6	ns
	Cough	0.8	0.3–2.1	ns

OR, odds ratio; CI, confidence interval.

cutoff value, we used multinomial analysis to investigate determinants of both slightly elevated (12–15 ppb) and high (> 15 ppb) FE_{NO}. The latter limit of FE_{NO} corresponds approximately to +3 SD of the distribution in healthy nonsmoking subjects, determined with similar technique and expiratory flows (20). Slightly elevated FE_{NO} was found to be associated with sputum eosinophilia, but not significantly with asthma, atopy, or BHR. This finding may suggest that slightly elevated FE_{NO} levels do not have any diagnostic value for asthma. Indeed, modest elevations of FE_{NO} have been reported to be associated in many inflammatory disorders, e.g. with chronic rhinitis, mild upper airway respiratory tract infections and atopy *per se* (6, 7, 26). On the contrary, asthma was the most important explanatory factor for high levels of FE_{NO}. However, the number of cases with high FE_{NO} was less than that with lung function changes diagnostic for asthma, suggesting low sensitivity of this grade of FE_{NO} elevation.

By using stepwise logistic regression analysis, clinical asthma and airway eosinophilia were the most important explanatory variables for high FE_{NO}. The relationship of FE_{NO} with mucosal eosinophilic inflammation has been shown in patients with asthma (4, 5), but the present study shows that this association prevails also in a broader range of patients with symptoms suggesting asthma. It is possible that some of the symptomatic patients showing sputum eosinophilia and increased FE_{NO}, but failing to demonstrate lung function changes diagnostic for asthma, may have a milder state which we have referred as 'asthma-like inflammation' (27). Similar patients have been shown to present with increased levels of FE_{NO} (28). Furthermore, these patients, like those with clinical asthma, are likely to respond favorably with inhaled corticosteroids (27).

Airway inflammation is one of the primary manifestations of asthma (29), and characteristically, eosinophils can be found in the airway mucosa both in atopic and nonatopic asthmatics (9, 13). Some investigators have found many similarities in the immunopathology of intrinsic and extrinsic asthma (10–12). These studies on bronchial biopsies have shown locally enhanced expression of Th2-type cytokines even in patients with nonatopic asthma, similar to that seen in patients with extrinsic type of asthma. Up-regulation of expression of iNOS may occur as a result of proinflammatory cytokines involved in asthmatic inflammation, common to both phenotypes of asthma. On the contrary, there are also reports that have described distinct patterns of inflammatory profile in allergic and nonallergic asthma (30). These findings could explain the differences of FE_{NO} levels between atopic and nonatopic asthmatics and subjects reported previously (6, 8, 23, 24). Accordingly in the present study, atopy was a significant factor, although not the strongest one, in explaining variation of FE_{NO} in the patients with symptoms suggesting asthma. The present results also suggest that the relationship between atopy and increased levels of FE_{NO} is based on the predisposition of eosinophilic airway inflammation, and when the latter is accounted for, atopy *per se* does not have any independent effect.

We conclude that in patients with symptoms suggesting asthma, slightly elevated and high levels of FE_{NO} are associated with sputum eosinophilia, whereas asthma is significantly associated only with high levels of FE_{NO}, irrespective of atopy. The results suggest that FE_{NO} is primarily a marker of airway eosinophilia, and that only high values of FE_{NO} may be useful to identify patients with atopic or nonatopic asthma.

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