

A comparison of exhaled nitric oxide and induced sputum as markers of airway inflammation

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Background: Exhaled nitric oxide (ENO) has been proposed as a noninvasive marker of airway inflammation in asthma.

Objective: We investigated the relationships among ENO, eosinophilic airway inflammation as measured by induced sputum, and physiologic parameters of disease severity (spirometry and methacholine PC₂₀). We also examined the effect of corticosteroid treatment and atopy on ENO levels and eosinophil counts in induced sputum.

Methods: Measurements were taken on one day in 22 healthy nonatopic subjects, 28 healthy atopic subjects, 38 asthmatic subjects not taking inhaled steroids, 35 asthmatic subjects taking inhaled steroids, and 8 subjects with eosinophilic bronchitis without asthma.

Results: ENO levels showed significant but weak correlations with eosinophil differential counts in the steroid-naïve asthmatic and healthy atopic groups ($r_s < 0.05$). ENO levels were significantly lower in the asthmatic subjects taking steroids compared with the asthmatic subjects not taking steroids, despite there being no difference in the sputum cell counts, and a tendency to increased airflow limitation. ENO levels and sputum eosinophil counts were equally good at differentiating from steroid-naïve asthmatic subjects. ENO levels were consistently raised in subjects with eosinophilic bronchitis without asthma. Atopy had no effect on ENO levels in the healthy subjects.

Conclusion: We conclude that ENO is likely to have limited utility as a surrogate clinical measurement for either the presence or severity of eosinophilic airway inflammation, except in steroid-naïve subjects. (*J Allergy Clin Immunol* 2000;106:638-44.)

Key words: Exhaled nitric oxide, sputum, asthma, eosinophilic bronchitis

Measurement of exhaled nitric oxide (ENO) has been considered as a marker of airway inflammation in airway diseases in general and asthma in particular. The origin of ENO is uncertain, but it is produced from epithelial cells and from various inflammatory cells, including eosinophils.¹ In asthma its production is possibly stimulated by upregulation of inducible nitric oxide synthase by proinflammatory cytokines. ENO levels are also raised in a

Abbreviations used

ENO: Exhaled nitric oxide
ICS: Inhaled corticosteroid
ppb: Parts per billion
SVC: Slow vital capacity
VC: Vital capacity

variety of other airway conditions associated with inflammation, such as viral infection,² bronchiectasis,³ and lung transplant rejection.⁴ Although there were initial problems with the standardization of measurements,⁵ there is now a better understanding of how to measure ENO from the lower respiratory tract without the influence of confounding factors.⁶ The apparatus is expensive, but the measurements are easy to perform.

Although ENO has been proposed as a marker of airway inflammation⁷ for some time, only recently has work been published on how it relates to direct measures of inflammation in bronchial biopsy specimens, bronchoalveolar lavage fluid, or sputum samples. Instead, most of the earlier studies of ENO in asthma examined associations with either physiologic indices, such as methacholine PC₂₀,^{8,9} or nonvalidated symptom scores,^{9,10} which can be unrelated to airway inflammation. The correlations that have been found between ENO and direct measures of airway inflammation, although often highly significant, have been of relatively small magnitude¹¹ and have recently been interpreted as showing that ENO measurement provides different information on airway inflammation compared with other more direct methods.^{12,13}

We have therefore investigated the relationship between ENO and sputum eosinophils in healthy subjects with and without atopy and patients with asthma and eosinophilic bronchitis without asthma and the ability of the two measurements to distinguish these groups from healthy subjects. We also compared these two parameters with airway function (as indicated by FEV₁ percent predicted, FEV₁/slow vital capacity [SVC], and methacholine PC₂₀) and with possible factors influencing the measurements, specifically inhaled corticosteroid (ICS) treatment and atopy.

METHODS

Subjects

One hundred thirty-one adult subjects were recruited from patients attending the Firestone Regional Chest and Allergy Clinic and by advertisement (Table I). There were 5 groups. Healthy subjects had

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TABLE I. Subject characteristics by group

	Healthy nonatopic	Healthy atopic	Asthma (no steroid)	Asthma (inhaled steroid)	Eosinophilic bronchitis without asthma
No.	22	28	38	35	8
Sex, M	11	12	15	14	4
Age, y	36 (13)	37 (12)	33 (15)	46 (15)	44 (10)
Atopic, n	0	28	35	27	5
FEV ₁ , % predicted	104 (11)	99 (13)	86 (17)	78 (17)	100 (13)
FEV ₁ /VC	0.79 (0.05)	0.79 (0.07)	0.72 (0.12)	0.67 (0.12)	0.79 (0.09)
PC ₂₀ , mg/mL	57.1 (103)	36.8 (92)	1.54 (2.47)	1.21 (3.92)	28.3 (90)
Budesonide equivalent dose, µg	0	0	0	800 (3160)	0 (350)

Values are expressed as mean (SD), except for PC₂₀, which is expressed as geometric mean (SD), and budesonide equivalent dose, which is expressed as median (interquartile range).

TABLE II. ENO and sputum results in individual and combined groups

	Healthy nonatopic	Healthy atopic	Asthma (no steroid)	Asthma (inhaled steroid)	Eosinophilic bronchitis without asthma
ENO, ppb	9 (7)	11 (6)	39 (43)	17 (12)	65 (92)
TCC, ×10 ⁶ cells/g	1.5 (2.4)	1.9 (2.8)	2.8 (3.5)	3.2 (5.5)	3.8 (4.1)
Viability, %	78 (26)	78 (24)	63 (24)	71 (16)	71 (20)
Neutrophil, %	37.0 (35.4)	38.5 (37.0)	29.8 (46.6)	38.6 (32.3)	31.3 (29.7)
Eosinophil, %	0.0 (0.3)	0.3 (1.0)	1.9 (9.6)	0.6 (3.8)	13 (23.5)
Macrophage, %	62.3 (31.3)	57.3 (36.2)	57.9 (48.1)	49.9 (26.8)	43.1 (34.0)
Lymphocyte, %	0.0 (0.5)	0.0 (1.0)	0.8 (1.0)	1.0 (1.2)	0.8 (1.7)
Bronchial epithelial cells, %	0.3 (1.1)	0.3 (1.2)	0.0 (0.5)	0.0 (0.5)	0.1 (0.3)

Values are expressed as median (interquartile range). In all groups squamous cell contamination of sputum was less than 5%.
TCC, Total cell count.

no symptoms, an FEV₁/vital capacity (VC) of 70% or greater, and a methacholine PC₂₀ of greater than 16 mg/mL and were divided into those with and without atopy on the basis of positive skin prick test results to one or more common allergen extracts. Asthma was identified by the presence of symptoms of wheeze, breathlessness, or cough in the last year plus methacholine airway hyperresponsiveness with a PC₂₀ of less than 8 mg/mL if the FEV₁/VC was 70% or greater or an improvement of the FEV₁ from predicted of 15% or greater after 200 µg of inhaled salbutamol if the FEV₁/VC was less than 70%. The asthmatic subjects were divided into those who had not received treatment with ICSs in the previous month and those receiving regular ICS therapy. Eosinophilic bronchitis without asthma was identified by cough in the last year, with an FEV₁/VC of 80% or greater, a methacholine PC₂₀ of greater than 16 mg/mL, and an induced sputum eosinophil count of 5% or greater of the total nonsquamous cell count, which was chosen as being well above the 90th percentile for sputum eosinophils.¹⁴ Current smokers were excluded from the study because smoking is known to reduce ENO levels,¹⁵ but exsmokers of greater than 1 year were included. No subjects had symptoms of an upper respiratory tract infection in the 4 weeks preceding the study or other complicating respiratory disease. Approval for the study was granted by the St Joseph's Hospital Research Committee, and written informed consent was obtained from all the subjects.

Study design

This was a descriptive study. Subject characteristics were obtained, and spirometry was performed; if the FEV₁/VC was 70% or greater, a methacholine test was carried out, and at the end of the test, 200 µg of salbutamol was inhaled. If the FEV₁/VC was less than 70%, 200 µg of salbutamol was inhaled, and after 15 minutes, spirometry was repeated. In either case, after another 15 minutes, ENO was measured and induced sputum was collected.

Procedures

Subject characteristics were documented by questionnaire. Allergy skin tests were performed by using the modified prick technique described by Pepys¹⁶ with negative and positive controls and 12 common allergen extracts. Spirometry was performed by using a KoKo-Trek spirometer (Trudell Medical, London, Ontario, Canada), according to American Thoracic Society criteria,¹⁷ and reference values were taken from the study of Crapo et al.¹⁸ Methacholine inhalation tests were carried out by the method described by Juniper et al.¹⁹

Sputum was induced with an aerosol of hypertonic saline by using the technique described by Pin et al²⁰ and subsequently modified by Pizzichini et al²¹ and was processed within 2 hours of expectoration, as described by Pizzichini et al.²² Briefly, the selected sputum was treated with dithiothreitol followed by PBS to break up the mucus and disperse the cells and then filtered to remove remaining mucus and debris. A total cell count was determined in a hemocytometer, cell viability was assessed by staining with trypan blue, cytospin preparations were made and stained with Wright's stain, and a differential cell count was carried out on 400 nonsquamous cells. The 90th percentile of normal cell counts, determined from a previous study from our laboratory, are a total cell count of less than 9.73 × 10⁶/g, a neutrophil count of less than 65% or 4.86 × 10⁶/g, an eosinophil count of less than 1.1% or 0.042 × 10⁶/g, and a macrophage count of 86% or 4.86 × 10⁶/g.¹⁴

ENO was measured by using a rapid linear-response chemiluminescence analyzer (Sievers 240, Boulder, Colo), according to a previously described and validated method.⁵ The subject inhaled to total lung capacity through a carbon filter (Survivair Division, USD Corporation, Santa Anna, Calif) to remove nitric oxide in ambient air and immediately exhaled through a high resistance generated by

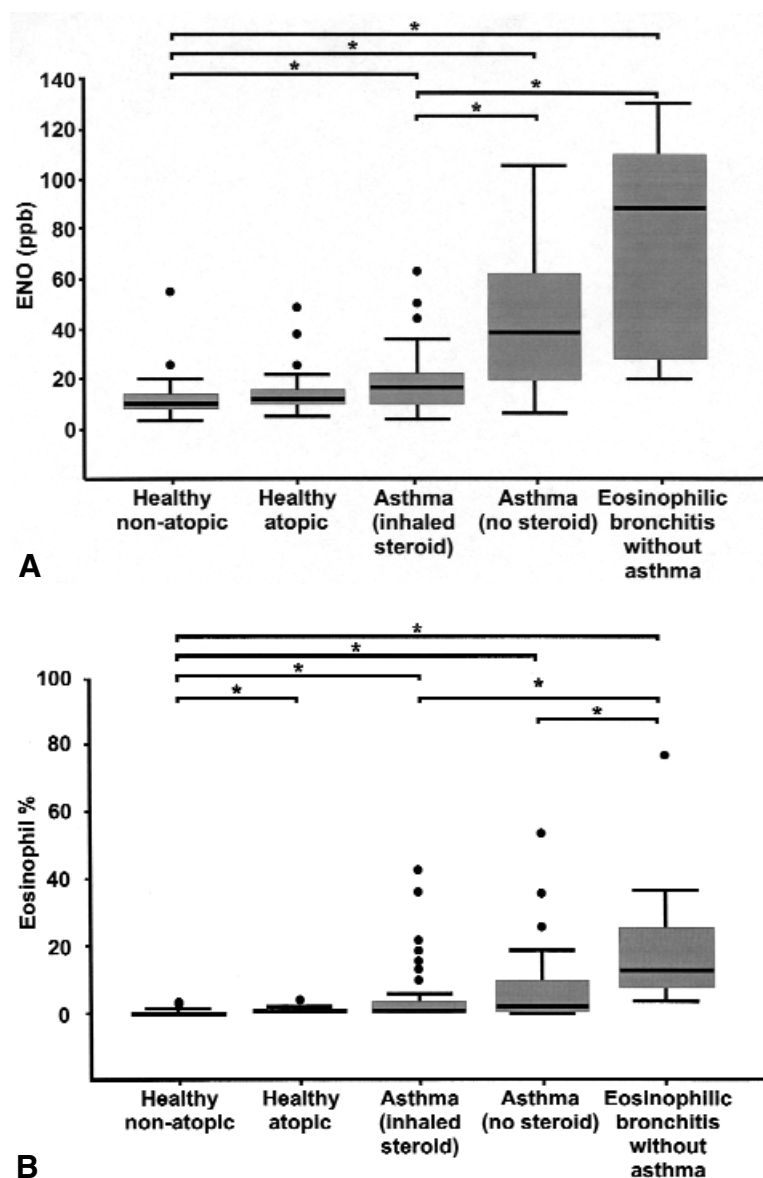


FIG 1. Box plots for ENO levels and eosinophil percentages by subject groups. The *thick bar* is the median value, and the *shaded box* represents the interquartile range. Outlying points beyond the inner fences are shown individually. Asterisks indicate significant differences ($P < .01$) between groups. There are significant differences in ENO levels between the healthy nonatopic group and the asthma and eosinophilic bronchitis groups and between the asthmatic groups with and without treatment. There was no significant difference in sputum eosinophil percentages between the asthmatic groups.

a 16-G standard medical needle. Subjects were asked to maintain a constant mouth pressure of 28 cm of water by observing a pressure gauge placed distal to the mouthpiece. This produced a fixed flow of 45 mL/s. ENO values were displayed on an XYY chart recorder (Omegaline; Omega Engineering, Stamford, Conn). Three values were taken with less than 10% variation, and the ENO level was recorded as the mean plateau value of the individual recordings.

Analysis

Results are reported as median (interquartile range) unless otherwise specified. All the statistical tests were two-sided, with significance being accepted at the 95% level. Nonparametric tests were used throughout because all the sputum values and the ENO values were

nonnormally distributed. Differences in the results of the physiologic tests, sputum cell indices, and ENO measurements from the groups with asthma not taking steroids, those with asthma taking inhaled steroids, those with eosinophilic bronchitis without asthma, healthy subjects with atopy, and nonatopic healthy subjects were analyzed by using the Kruskal-Wallis ANOVA test. Apparent differences between the individual groups were examined by using the Mann-Whitney U test for the sputum indices and ENO measurements and by using the Student t test for the physiologic tests. Cell counts were analyzed both as absolute values and as percentages of the total nonsquamous cell count. Receiver operating characteristic curves were constructed for ENO levels and eosinophil percentage counts in the asthma groups and the healthy group with atopy to test the ability of the two mea-

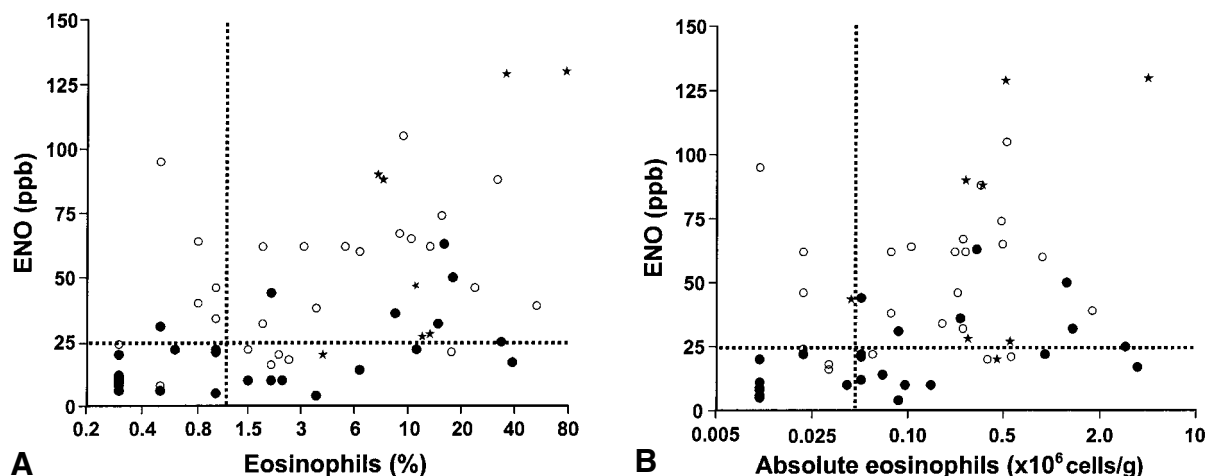


FIG 2. Scatter plot of ENO levels and differential and absolute eosinophil counts in the subjects with asthma not treated with steroids (*open circles*), those with asthma treated with inhaled steroids (*closed circles*), and those with eosinophilic bronchitis without asthma (*asterisks*). The *dashed lines* indicate the 90th percentile for the measurement in healthy subjects ($n = 20$). There is a wide scatter of ENO levels throughout the range of eosinophilia measured.

measurements to distinguish these groups from the healthy nonatopic group. The normal group was also used to establish an upper limit of the normal range (90th percentile) for ENO. Correlations between ENO values and sputum cell counts and the physiologic data were examined both in the asthma and eosinophilic bronchitis groups together and in each individual group by using the Spearman rank test. Missing data were excluded listwise.

RESULTS

We compared ENO levels and sputum cell counts in subjects with asthma, subjects with eosinophilic bronchitis without asthma, and healthy subjects (Tables I and II). There were significant differences in ENO levels, eosinophil percentages, absolute eosinophil counts ($\times 10^6/g$; $P < .001$), macrophage percentages ($P = .023$), and lymphocyte percentages ($P = .001$) between the groups, as well as for FEV_1 percent predicted, FEV_1/SVC , and PC_{20} ($P < .001$).

Effect of ICS therapy on ENO and sputum eosinophils

ENO levels were significantly lower in asthmatic subjects taking ICSs compared with asthmatic subjects not taking ICSs ($P < .001$) and patients with eosinophilic bronchitis without asthma ($P < .001$), who had the highest level of ENO, despite 3 of them taking ICSs (Table II, Fig 1). However, sputum eosinophil counts in asthmatic subjects taking ICSs, although significantly lower than those in patients with eosinophilic bronchitis but without asthma, was not different from those of asthmatic subjects not taking ICSs.

Modest but significant correlations were observed between ENO levels and eosinophil percentage counts of asthmatic subjects not taking ICSs but not in asthmatic subjects taking ICSs (Table III, Fig 2). Similarly, significant correlations were observed between ENO levels, eosinophil percentages, FEV_1 , and FEV_1/SVC only in

the group of asthmatic subjects not taking ICSs. They had significantly greater FEV_1 percent predicted ($P < .05$) and FEV_1/SVC ($P = .052$) compared with those taking ICSs. However, the PC_{20} methacholine was significantly correlated with the ENO level only in asthmatic subjects taking ICSs. There were no significant correlations between ENO levels or sputum cell counts with the dose of ICS.

Using an ENO level of 23 parts per billion (ppb) as the upper limit of normal (mean, 12 ppb; SD, 11 ppb; 10th and 90th percentiles of 6 and 23 ppb, respectively, in healthy nonatopic subjects), there were 6 subjects (all asthmatic subjects not taking ICSs) with a high ENO level and normal eosinophil percentage, none of whom consistently showed other high sputum differential cell counts.

Effect of atopy on ENO and eosinophils

ENO levels were not different in the atopic and nonatopic healthy subjects (Table II). Eosinophil percentage counts were also normal in both groups, with atopic subjects having a statistically significantly higher value. There was a significant correlation between ENO levels and eosinophil percentages in the healthy atopic subjects (Table III).

Sensitivity and specificity of ENO and eosinophils in diagnosing asthma and atopy and the effects of ICSs

ENO levels of 17.1 ppb had a sensitivity of 81% and a specificity of 90%, and eosinophil counts of 0.3% had a sensitivity of 78% and specificity of 85% for the diagnosis of asthma when the asthmatic patients were not taking ICSs (Fig 3). The sensitivity and specificity of these measurements for diagnosing asthma were lower when the patients were taking ICSs (sensitivity and specificity of ENO at 15.1 ppb of 51% and 86%, respectively, and

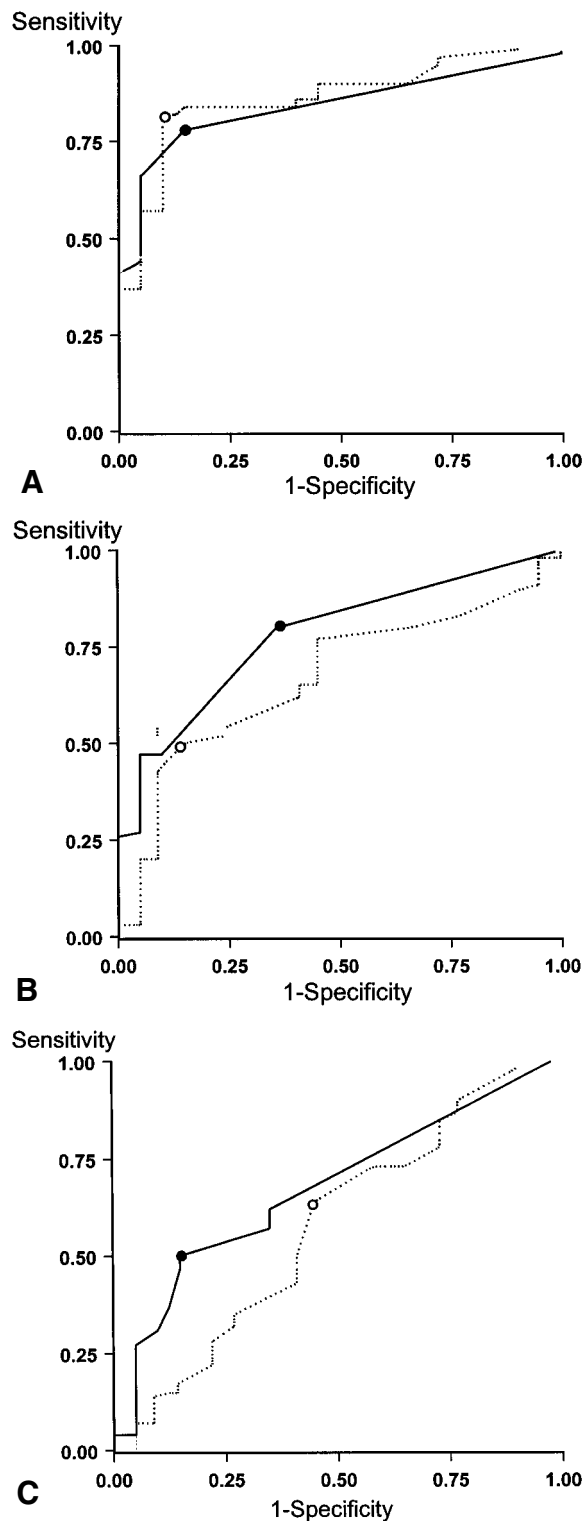


FIG 3. Receiver operating characteristic curves for ENO levels (dashed line) and eosinophil percentages (solid line) for patients with asthma not treated with steroids (**A**), patients with asthma treated with inhaled steroids (**B**), and healthy atopic subjects (**C**). Open circles indicate optimum cut-off points for ENO levels, and filled circles indicate optimum cut-off points for eosinophil percentages. The two tests performed equally well at differentiating steroid-naïve asthmatic subjects from healthy subjects.

79% and 65% respectively, for eosinophil percentage, with a cut-off point of 0.08%). As tests to distinguish atopic from nonatopic healthy subjects, eosinophil percentage (cut-off point, 0.33%; sensitivity, 78%; specificity, 85%) performed somewhat better than ENO (cut-off point, 9.0 ppb; sensitivity, 64%; specificity, 54%).

DISCUSSION

The results of this cross-sectional study show weak correlations between ENO and sputum eosinophils in atopic healthy and asthmatic subjects taking and not taking steroids. Lower levels of ENO than would be expected from the degree of sputum eosinophilia and airway narrowing were found in subjects treated with steroids. High levels of ENO were found in some normal and asthmatic subjects with normal sputum eosinophils. ENO measurement was capable of distinguishing subjects with asthma from healthy subjects, provided that the subjects were not taking inhaled steroids. The findings suggest that the measurement of ENO, at one moment in time, has some utility in indicating the presence and intensity of eosinophilic bronchitis in asthma, particularly when the patient is not being treated with ICSs. Additionally, ENO measurement may be a useful test in the diagnosis of eosinophilic bronchitis without asthma, but treatment with steroids in asthma would likely limit this utility. They also indicate that a raised level of ENO is not specific for eosinophilic airway inflammation and may reflect other facets of airway inflammation not apparent from sputum cell counts or physiologic testing.

The present cross-sectional study mimics how measurements would be applied in practice to investigate the presence of airway inflammation. The methods used to measure ENO levels and sputum cell counts have been shown to be reproducible (reliable)^{5,22} and responsive.²³⁻²⁶ They can therefore be considered to be accurate. Similar correlations between ENO and sputum eosinophils have been observed before,^{11,27} but other studies have shown conflicting negative results.^{12,13} The present study is one of the largest ones to examine these correlations. The receiver operating characteristic curves for both sputum eosinophilia and ENO suggest that in the subjects not taking inhaled steroids, both tests could perform equally and reasonably well as diagnostic tools to confirm the presence of asthma. However, the raised levels of ENO observed in some subjects with normal sputum eosinophils questions whether ENO is more sensitive than sputum for indicating airway eosinophilia or whether it is less specific, measures other aspects of airway disease or inflammation aside from eosinophilia, or both. The latter would appear to be correct. Normal eosinophil counts in sputum seems to correlate with few if any eosinophils in bronchial biopsy specimens,²⁸ and sputum eosinophilia precedes clinical deterioration.²⁹ These observations suggest that sputum eosinophilia is a sensitive indicator of airway eosinophilia and is of clinical relevance. Furthermore, increased levels of ENO have been observed in other conditions characterized by neutrophilic, noneosinophilic inflammation, such

TABLE III. Correlations between ENO levels and eosinophil counts and physiologic parameters in subjects with asthma and combined eosinophilia

Group		ENO	Eosinophils (%)	Eosinophils (10 ⁶ /g)
Asthma (no steroid)	ENO	—	0.47*	0.45†
	PC ₂₀	−0.16	−0.02	−0.05
	FEV ₁ % predicted	−0.36*	−0.40*	−0.45*
	FEV ₁ /VC	−0.38*	−0.60†	−0.61†
Asthma (inhaled steroid)	ENO	—	0.31	0.38*
	PC ₂₀	−0.35*	−0.24	−0.22
	FEV ₁ % predicted	−0.22	−0.33	−0.32
	FEV ₁ /VC	−0.29	−0.3	−0.3
Eosinophilic bronchitis without asthma	ENO	—	0.55	0.19
	PC ₂₀	−0.39	0.04	0.71
	FEV ₁ % predicted	−0.38	−0.29	0.26
	FEV ₁ /VC	−0.33	−0.12	0.6
Healthy atopic	ENO	—	0.47*	0.48*
	PC ₂₀	−0.27	−0.14	−0.16
	FEV ₁ % predicted	−0.14	−0.06	0.16
	FEV ₁ /VC	−0.11	−0.42*	0.32
Healthy nonatopic	ENO	—	0	0.04
	PC ₂₀	−0.14	−0.18	−0.16
	FEV ₁ % predicted	0.32	0.41	0.44
	FEV ₁ /VC	0.29	0.21	−0.14

Values are expressed as r_s , and significant correlations are shown in bold.

* $P < .05$.

† $P < .01$.

as respiratory virus infection and bronchiectasis, illustrating its lack of specificity for eosinophilia.^{2,3}

The lower levels of ENO in subjects with asthma treated with ICSs have been observed before. The levels have been observed to fall after an increase in steroid therapy^{12,13,23,24} and rise after a reduction of treatment.⁹ The findings have been interpreted as evidence that ENO is a marker of airway inflammation, possibly of steroid-sensitive mechanisms other than airway eosinophilia. In the present study, which had large subject numbers compared with previous studies, there was a similar degree of sputum eosinophilia and slightly less severe airflow limitation in the steroid-naïve subjects compared with the steroid-treated subjects with asthma. There are two possible explanations of the disparity between these observations and the lower levels of ENO in this group. First, ENO may be more sensitive to inhaled steroids than sputum eosinophilia, as has been previously suggested.³⁰ Second, the lower levels of ENO may be a result of downregulation of inducible nitric oxide synthase alone by steroids, rather than an effect on airway inflammation per se. We did not find any correlation between steroid dose and ENO levels or sputum cell counts in the steroid-treated group, although the study was not designed to look at the complex question of dose-response relationships of each measurement. If ENO measures different aspects of inflammation in asthma, it may require more subtle testing to establish what is being measured above and beyond the conventional means used in this and previous studies, namely bronchial biopsy, lavage, and sputum examination.

The observation of a correlation between ENO and airway hyperresponsiveness in the steroid-treated group

but in no other group is of uncertain significance. Although some studies have not shown a relationship between these two parameters,²⁷ previous studies that have reported significant correlations have done so in steroid-naïve subjects only.^{8,13} Furthermore, it is unlikely that there is a direct relationship between nitric oxide and airway hyperresponsiveness because attempts at manipulation of nitric oxide with inducible nitric oxide synthase inhibitors have not affected airway hyperresponsiveness in a predictable fashion.³¹ It therefore seems likely that ENO and hyperresponsiveness are not measuring the same aspects of airway disease.

The documentation of high levels of ENO in eosinophilic bronchitis without asthma is a new observation. This condition, which usually presents with a chronic cough, can be asymptomatic.³² It was seen in two of the subjects considered to be healthy in whom ENO levels were also raised. This raises the possibility that ENO might be used as a diagnostic tool in eosinophilic bronchitis without asthma, but this requires investigation of the sensitivity and specificity of ENO in chronic cough. We also found, as has been previously reported,¹⁴ a small difference between atopic and nonatopic healthy subjects for eosinophil counts, although ENO levels were not different between the two healthy groups despite significant correlations between the two parameters in the atopic group. These different observations in the different groups again suggest a complexity of relationship between airway inflammation and ENO.

The reason for wanting to measure ENO levels or sputum cell counts in clinical practice is that they might be useful to guide anti-inflammatory treatment. Eosinophilic

airway inflammation and the clinical features associated with it are reduced by corticosteroid treatment^{25,26,33}; there is increasing evidence that noneosinophilic inflammation is not improved.³³⁻³⁵ ENO levels are potentially of use in the context of steroid-naïve subjects to diagnose the presence of airway inflammation. However, if they can be reduced by steroid treatment by mechanisms that have an unclear relationship to airway inflammation, and if they can be elevated by noneosinophilic airway inflammation, their clinical utility will be limited.

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