

LETTER TO THE EDITOR

EXHALED NITRIC OXIDE AS A MARKER OF LUNG INVOLVEMENT
IN CROHN'S DISEASE

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Crohn's Disease is an inflammatory bowel disease associated with a variety of systemic manifestations, including large and small airway involvement. The latter is most often a subclinical one, and requires expensive and invasive diagnostic approaches. Nitric Oxide (NO) can be detected non-invasively in the exhaled air (eNO) and be considered as a surrogate marker of airway inflammation. eNO tested at multiple expiratory flows can be used to distinguish the alveolar concentration of NO ($C_{alv}NO$) from the total amount of fractional eNO (FeNO). The aim of our study is to compare FeNO and concentration of alveolar nitric oxide ($C_{alv}NO$) levels and to assess their relationship with pulmonary involvement in Crohn's patients differing in clinical stage and therapeutic regimens versus a group of healthy subjects. Thirty Crohn's patients not showing clinical evidence of pulmonary diseases and 21 non-smoking, non-atopic healthy controls were enrolled. FeNO (14.9 ± 10.2 ppb vs 10.1 ± 6.3 ppb, $p=0.049$) and $C_{alv}NO$ (4.4 ± 2.2 ppb vs 2.6 ± 1.9 ; $p=0.006$) values were found to be significantly higher in Crohn's patients than in healthy controls. Both FeNO and $C_{alv}NO$ correlated positively with the Crohn's Disease Activity Index. In conclusion, our results for FeNO and $C_{alv}NO$ confirm the presence of subclinical pulmonary involvement in Crohn's disease. eNO measurement may be of clinical value in the follow-up of Crohn's patients.

Crohn's disease (CD) is an inflammatory bowel disease (IBD) of unknown aetiology characterized by intestinal and extra-intestinal manifestations suggesting the presence of a systemic inflammatory disorder (1-2). The lungs may be also involved, which will worsen the overall prognosis (1, 3). Several reports have demonstrated that, nearly half of IBD patients show some degree of lung involvement (4-5), including small airway (6), parenchymal and pulmonary vascular disease as well as serositis (7-8). A number of studies have been focusing on pulmonary function tests (PFT) which in turn have revealed abnormalities such as

a decrease in lung diffusing capacity in nearly 57% of CD patients showing no radiological alterations of the chest (9). These abnormalities, which have also been described during clinical remission, are the likely consequence of a subclinical inflammatory reaction. This hypothesis draws additional support from the increased airway hyper-responsiveness that has been documented in asymptomatic IBD patients (10). Most of these patients are usually asymptomatic; in addition, the procedures necessary to prove pulmonary involvement are quite costly and complicated. Exhaled Nitric Oxide (eNO) can be detected non-invasively by using a

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chemiluminescence-based technique, and has been reported to be elevated in several airway inflammatory diseases and to be a marker of airway inflammation (11-12). Moreover, the use of a bi-compartmental model has recently made possible the observation that eNO tested at multiple expiratory flows can be used to distinguish the alveolar concentration of NO ($C_{\text{alv}}\text{NO}$) from the total amount of fractional eNO (FeNO) giving patho-physiological insights on the NO originated directly from the lung airways instead of total exhaled NO (14-15).

NO plays an important role in many physiological and pathological events involving the gastrointestinal tract (16), and is considered an important inflammatory mediator in IBD. Increased NO levels in aspirated colonic gases have been observed in patients with ulcerative colitis more commonly than in controls (16). The aim of the present study is to evaluate whether FeNO or $C_{\text{alv}}\text{NO}$ is increased in patients with active Crohn's disease compared to controls in relation to the disease activity, the CRP and the different treatment regimens of Crohn's disease patients

MATERIALS AND METHODS

Patients

We studied 30 in- and out-patients at different stages of CD who were admitted to the Gastroenterology Unit of the Department of Internal Medicine of Spedali Civili general hospital Brescia, Italy. Our diagnosis of CD was based on medical history, physical examination, and radiological, endoscopic and histological findings. Demographic data, clinical history, duration of disease, medications used, extent of disease were all recorded. Age-matched healthy subjects were then selected for the control group. The enrolled patients did not report symptoms or show signs of lung disease such as cough, sputum, dyspnoea, haemoptysis. Conditions possibly affecting eNO levels, namely cigarette smoking, atopy, respiratory tract infections over the previous 6 weeks, lung disease [asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, bronchiectasis, respiratory failure] were set as exclusion criteria. Upon inclusion in the study, each patient belonging to the CD group was treated topically and/or systemically with an individualized as well as constantly monitored and adjusted combination of anti-inflammatory drugs (mesalazine), biological drugs (infliximab, adalimumab), and immunosuppressant (methotrexate, azathioprine). Table I shows the different treatment regimens. All patients and controls gave their

written informed consent to participate in the study, which was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the local Ethics Committee.

Study design

This is an observational controlled study. On enrolment, all CD patients were examined and they filled in a questionnaire specially designed to detect the presence and extent of intestinal (abdominal pain, fever, frequency of evacuations, presence of blood during evacuations) and pulmonary (cough, sputum, dyspnoea, haemoptysis) symptoms. Demographic data, clinical history, macroscopic and histological evaluation of the disease, duration and severity of disease, medications used, and co-morbidities were also recorded. A blood sample was collected for CRP analysis on the day of the first eNO measurement. Twenty-one control subjects were then enrolled from healthy volunteers whose medical history and physical exam were negative for CD.

Disease activity

The Crohn's Disease Activity Index (CDAI) was used to establish disease activity. CD patients with scores below 150 were considered in clinical remission, while patients scoring over 150 were considered with active disease (150-250: mild activity; 250-350: moderate activity; > 350: severe activity) (17).

eNO measurement

eNO levels were assessed with a high-resolution chemiluminescence nitric oxide analyzer (Ecomedics AG CLD 77 AM; Ecomedics; Dürnten, Switzerland) with a 0.06 part per billion (ppb) detection limit and an upper measuring range limit of 100 ppb. Measurements were performed at different flows in accordance with American Thoracic Society recommendations and by using a standardized procedure for the online measurement of FeNO in adults (18). Exhaled NO was measured at multiple flow rates of 50, 100 and 200 ml/s. For each flow rate the mean value of three measurements was used. Single exhaled NO fraction (FeNO) was measured at a flow rate of 50 ml/s. A mathematical approach based on a two-compartmental model was used to differentiate NO generated in the airways ($C_{\text{alv}}\text{NO}$). (15) The CLD 77 analyzer was calibrated at 0 ppb and 100 ppb in accordance with the manufacturer's recommendations. The analyzer was calibrated daily using a certified nitric oxide mixture (96 ppb) in nitrogen (Messer S.p.A.; Collegno-TO, Italy). Ambient air was monitored for nitric oxide concentration prior to data collection; no measurements were taken with ambient nitric oxide above 10 ppb. In light of the data we previously obtained from a large cohort of healthy

individuals, we considered normal all FeNO readings below 11 ppb at a constant flow rate of 50 mL/s (19-21). All tests were conducted at the same hour in the morning; all patients had to be on an empty stomach and those treated with biological drugs (infliximab, adalimumab) were carefully examined prior to each injection.

CRP collection

All CRP values were determined from blood samples. In light of the different methods and cut-off levels involved in the process, we expressed the data in the form of a ratio (observed value / upper limit of normal according to manufacturer).

Outcome variables

The main outcome variables of this study consisted in the comparison of FeNO and C_{alv} NO levels between CD patients and controls, and in the correlation between these levels and the CDAI. The secondary outcome variables were the relationship between FeNO and C_{alv} NO values and CRP values, and the effect of different therapeutic regimens for CD on FeNO and C_{alv} NO.

Statistical analysis

Descriptive statistics were used to summarize the demographic characteristics of the patients. Continuous data were summarized with the mean and standard deviation (SD) if normally distributed. Continuous variables were compared by Student's *t* test, and when any given patient was compared both in the induction and in the maintenance phase of the treatment, a pair analysis was conducted. Correlations were evaluated by linear regression, a *p* value < 0.05 being considered significant. Data analysis was conducted using statistical software (GraphPad Prism 5).

RESULTS

Thirty CD patients, 15 males and 15 females (mean age 43.10 ± 14.6 yrs, body mass index (BMI) 25.7 ± 4.1) and 21 healthy controls (9 males, 12 females, mean age 40.1 ± 13.2 yrs, BMI 21.6 ± 2.3) were enrolled. There were no anthropometric differences between the 2 groups. Patients in the CD group showed no clinically detectable pulmonary involvement. The mean CDAI of the patients studied was 160.7 ± 109.4 . The characteristics of the two groups are summarized in Table I.

FeNO levels at different flow rates

In the 30 CD patients C_{alv} NO was measured

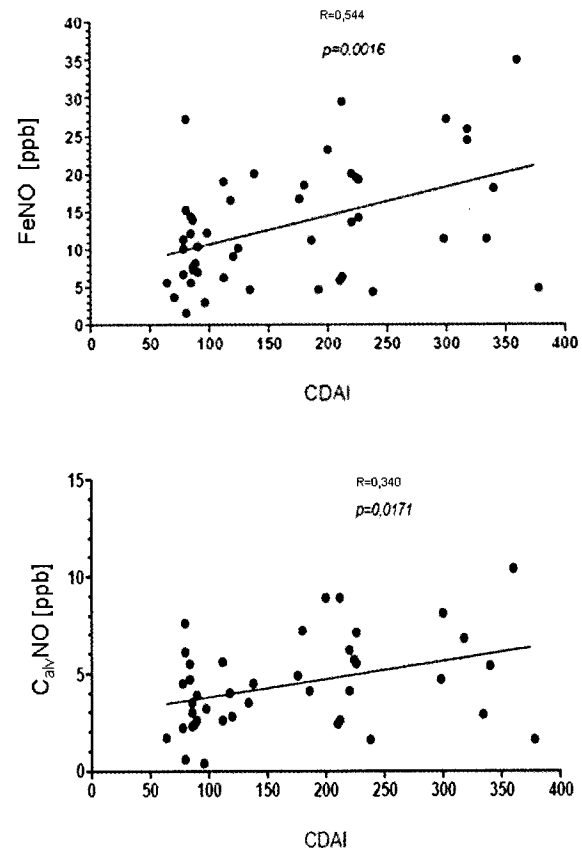


Fig. 1. Correlation between *e*NO (ppb) and the disease activity in CD. There are significant correlations between FeNO and C_{alv} NO with CDAI.

on 47 occasions and FeNO on 56 occasions. The mean FeNO and C_{alv} NO levels were significantly higher in the CD group than in the healthy controls (respectively 14.93 ± 10.26 ppb vs 10.13 ± 6.37 ppb; $p=0.049$ and 4.43 ± 2.27 ppb vs 2.62 ± 1.97 ppb; $p=0.006$).

*e*NO and disease activity

The CDAI was calculated on 48 occasions with the following results: CDAI below 150 on 26 (54%), between 150 and 250 on 14 (29%), between 250 and 350 on 6 (12%), and above 350 on 2 (4%) occasions. Fifty-six paired observations of CD patients revealed a significant correlation between FeNO ($p=0.017$) and the CDAI and C_{alv} NO ($p=0.002$) and the CDAI (Fig. 1), showing a moderate ($\rho = 0.54$) and weak ($\rho = 0.34$) correlation respectively. No correlation

Table I. Characteristics of patients with Crohn's Disease (CD) and controls.

	CD	Controls
Number	30	21
Gender (m/f)	15/15	9/12
Mean age - yrs - (SD)	43.1 (14.6)	40.1 (13.2)
BMI - Kg/m ² - (SD)	25.7 (4.1)	21.6 (2.3)
Smokers	0	0
Patients treated with mesalazine (n)	5	N.A.
Patients treated with biological drugs (n)	20	N.A.
Patients treated with immuno-soppressors (n)	5	N.A.
Mean FENO (50 mL/s) - ppb - (SD)	14.9 (10.2)	10.1 (6.3)
Mean C _{alv} NO - ppb - (SD)	4.4 (2.2)	2.6 (1.9)
Mean activity index (SD)	160.7 (109.4)	

Values are given as mean $\pm$ standard deviation (SD), for CD the Crohn's disease activity index (CDAI) is shown.

was observed between FeNO and C_{alv}NO values with CRP levels.

FeNO and localization of the disease

We carried out 17 C_{alv}NO measurements on patients with isolated small bowel disease and 15 FeNO measurements on patients with isolated large bowel disease. We did not observe any significant differences in these data between the patients with small-intestine CD and those suffering from large-intestine CD (FeNO: 15.48 $\pm$ 6.56 ppb vs 17.06 $\pm$ 9.70 ppb, in small and large bowel disease respectively; $p=0.560$, C_{alv}NO 4.95 $\pm$ 2.16 ppb vs 5.06 $\pm$ 2.43 ppb in small and large bowel disease respectively; $p=0.865$)

eNO levels as a function of the therapeutic approach to CD

Mean bronchial eNO was significantly different

depending on the type of drug/drugs used in the treatment of CD. FeNO was 7.88 $\pm$ 4.19 ppb in the patients treated with the anti-inflammatory drug mesalazine (n=5) compared to 14.35 $\pm$ 7.88 ppb in those treated with the biological drugs infliximab or adalimumab (n=20) and to 20.42 $\pm$ 11.45 ppb in those treated with the immunosuppressant azathioprine or methotrexate (n=5) ($p=0.04$).

There was no significant difference in mean C_{alv}NO between patients treated with the anti-inflammatory drug mesalazine (n=5, 3.33 $\pm$ 0.81 ppb), those treated with infliximab or adalimumab (n=20, 4.61 $\pm$ 2.41 ppb) and those treated with azathioprine or methotrexate (n=5, 4.25 $\pm$ 2.05 ppb).

No difference emerged in patients treated with biological drugs between the eNO readings obtained during the induction phase of treatment and those obtained during the maintenance phase (C_{alv}NO 4.46 $\pm$ 2.16 ppb vs 4.50 $\pm$ 2.50 ppb, $p=0.965$ and FeNO

14.31±5.85 ppb vs 13.89±8.42 ppb, $p=0.894$).

DISCUSSION

The results of our study show that eNO levels are significantly higher in CD patients than in controls, and that these levels are positively related to CDAI. Both findings suggest the presence of a subclinical, disease-activity-related inflammatory involvement of the lung. These results are in line with those of a previous study by Koek et al., (22) a study likewise designed to investigate the correlation between eNO levels and CD activity. The Authors found high levels of FeNO in CD patients, and established a negative correlation between CDA and pulmonary function indexes (forced expiratory volume in one second and forced vital capacity), and went on to suggest the presence of a link between bronchial inflammation and systemic disease activity in CD. Another recent study by Ozyilmaz et al. confirmed the correlation between FeNO levels and disease activity in patients with CD and ulcerative colitis (UC) (23). While these studies only considered FeNO levels (measured at 50 ml/s), and only contemplated one single exam per patient, the current study analyses eNO with multiple flow rates and at different stages of each patient's clinical and treatment courses. This approach enabled us to identify the relationship linking different types of eNO with clinical disease activity and therapeutic regimens. Both FeNO and C_{alv} NO values increased in agreement with the disease activity, suggesting a correlation between airway inflammation, and the clinical severity of the intestinal disease. The latter finding suggests an involvement of the alveolar sites and small vessel compartment of the lung in chronic inflammatory bowel disease, and is also likely to reflect overproduction of nitric oxide outside the lung, namely at the site of intestinal and extra-intestinal inflammation that characterize CD. Moreover, it is possible to hypothesize that the lung is able to eliminate the overproduction of nitric oxide at the intestinal level in patients with Crohn's disease, considering the lung as an organ able to expel gases carried in the blood. Thus, it is possible that exhaled NO reflects inflammatory bowel activity at distance. To better evaluate eNO levels in patients on treatment with different medications, all the CD patients were divided into three groups depending

on the type of treatment they were undergoing at the time of measurement. Our results clearly show that patients with mild disease treated with anti-inflammatory drugs (mesalazine) tend to have FeNO and C_{alv} NO values lower than those of patients under biological drugs such as infliximab and adalimumab or immunosuppressants such as azathioprine and methotrexate. Unfortunately, the data on hand provide no conclusive evidence as to whether, and to what extent, the low eNO levels observed in patients treated with mesalazine were due to the disease's mild activity or to the use of mesalazine itself. Results in the subgroup of CD patients treated with biological drugs were analyzed separately, relative to each therapeutic stage (induction and maintenance), and compared with those obtained in healthy subjects. The fact that both FeNO and C_{alv} NO levels proved equally high at both therapeutic stages suggests that a subclinical lung inflammation is present even during pharmacologically induced remission of the disease. In some patients, eNO was measured several times during the study period, but the comparison between the readings obtained from them did not show any significant variation (data not shown). Though fully aware of the relatively small size of the groups we studied, we nonetheless believe that further research is warranted in this area. Although the mechanisms leading to chronic airway inflammation in patients with CD are yet to be fully understood (24-25), eNO measurement has proven very useful in the non-invasive detection and severity assessment of CD-related pulmonary involvement, which qualifies it as a surrogate marker of pulmonary inflammation. Further research is warranted to compare eNO readings with results from other techniques such as the electronic nose and exhaled breath condensate tests (24-25). In conclusion, eNO shows great promise in assessing CD severity by revealing the presence and extent of CD-related subclinical airway inflammation.

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