Dissociation between exhaled nitric oxide and hyperresponsiveness in children with mild intermittent asthma

Michela Silvestri, Daniela Spallarossa, Elena Battistini, Vito Brusasco, Giovanni A Rossi

Abstract

***Background*—Bronchial hyperresponsiveness and airway inflammation are distinctive features of asthma. Evaluation of nitric oxide (NO) levels in expired air have been proposed as a reliable method for assessing the airway inflammatory events in asthmatic subjects. A study was undertaken to evaluate whether airway hyperresponsiveness is related to levels of exhaled NO.**

***Methods*—Thirty two steroid-naive atopic children with mild intermittent asthma of mean (SD) age 11.8 (2.3) years and 28 age matched healthy controls were studied to investigate whether baseline lung function or airway hyperresponsiveness is related to levels of exhaled NO. Airway responsiveness was assessed as the dose of methacholine causing a 20% decrease in forced expiratory volume in one second (FEV_1) from control (PD_{20} methacholine) and exhaled NO levels were measured by chemiluminescence analysis of exhaled air.**

***Results*—At baseline asthmatic children had significantly higher NO levels than controls (mean difference 25.87 ppb (95% CI 18.91 to 32.83); p<0.0001) but there were no significant differences in lung function parameters (forced vital capacity (FVC), FEV_1 (% pred), and forced expiratory flows at 25–75% of vital capacity ($FEF_{25–75\%}$)). In the asthmatic group exhaled NO levels were not significantly correlated with baseline lung function values or PD_{20} methacholine.**

***Conclusions*—These results suggest that levels of exhaled NO are not accurate predictors of the degree of airway responsiveness to inhaled methacholine in children with mild intermittent asthma.**

(*Thorax* 2000;**55**:484–488)

Keywords: airway inflammation; asthma; exhaled nitric oxide; methacholine; lung function

Divisione di Pneumologia, Istituto G Gaslini, Largo G Gaslini 5, 16148 Genoa, Italy
M Silvestri
D Spallarossa
E Battistini
G A Rossi

Cattedra di Fisiopatologia Respiratoria, Dipartimento di Scienze Motorie, Università di Genova, Genoa, Italy
V Brusasco

Correspondence to:
Dr G A Rossi
email: giovannirossi@ospedale-gaslini.ge.it

Received 31 August 1999
Returned to authors
22 December 1999
Revised version received
24 January 2000
Accepted for publication
8 March 2000

Airway inflammation and hyperresponsiveness are recognised as major characteristics of bronchial asthma.[1] Mediators released by inflammatory cells, mainly eosinophils and mast cells, have the potential to injure airway tissues, to increase bronchial hyperresponsiveness, and to induce the structural changes that lead to progressive loss of respiratory function.[1] Because of these observations, it has been suggested that, even in mild asthma, monitoring of airway inflammation and bronchial responsiveness may be useful for gauging the severity of the disease and the efficacy of anti-inflammatory treatment.[2]

Measurements of airway hyperresponsiveness to methacholine or histamine have become popular in clinical practice, epidemiology and research, even in the paediatric population.[3] However, these tests are time consuming and require levels of collaboration not always achievable in younger patients. Assessments of airway inflammation can be obtained invasively by bronchoalveolar lavage and bronchial biopsy or non-invasively by induced sputum,[4 5] but these methods are not always applicable in clinical routine, particularly in young children. Recently, the measurement of expired nitric oxide (NO) has been proposed as a non-invasive, simple, well tolerated test to assess airway inflammation in asthma.[6 7]

For practical purposes it would be important to know whether airway hyperresponsiveness can be predicted by biological markers of airway inflammation and vice versa. Studies using bronchoalveolar lavage, bronchial biopsy specimens, or induced sputum failed to show convincing correlations between airway hyperresponsiveness and inflammatory cell numbers.[8]

The purpose of the present study was to evaluate whether airway hyperresponsiveness to methacholine is predictable from measurement of exhaled NO levels in children with mild intermittent asthma.

Methods

SUBJECTS

Thirty two children (12 girls) of mean (SD) age 11.8 (2.3) years (range 6–15) referred to our outpatient clinic with a history of mild intermittent asthma[2] were studied. Allergic sensitisation to one or more allergens was demonstrated in all cases by a standardised skin prick test and RAST (Pharmacia Diagnostics AB, Uppsala, Sweden).[9] Eleven patients were sensitised to house dust mite only, one to pollen only, and 20 to house dust mite + other allergens (pollens, cat, moulds). All children were known to have a positive response to a methacholine inhalation challenge—that is, a decrease in forced expiratory volume in one second (FEV_1) equal to or greater than 20% from control after 1 mg methacholine. None of them was taking anti-asthmatic treatments other than short acting β_2 agonists on an as required basis which were avoided for 12 hours before the study.

Table 1 Characteristics of study subjects

	Controls	*Asthmatic patients*
Age (years)	11.6 (2.5)	11.8 (2.3)*
FVC	100.7 (15.7)	98.8 (11.9)*
FEV_1 (% pred)	103.5 (17.4)	97.0 (9.8)*
$FEF_{25-75\%}$	110.1 (24.7)	100.6 (19.3)*
NO (ppb)	4.1 (2.6)	30.0 (19.2)**
Methacholine (μg)	—	134.3***

*p<0.05; **p<0.01; ***p<0.001.

Twenty eight age and sex matched healthy children of mean (SD) age 11.6 (2.5) years, evaluated within a different study performed on schoolchildren, served as a control group. They had negative reactions to the standardised skin prick test and normal IgE serum levels in serum.[9] Parents or tutors of children were informed of the scope of the study and of the procedures involved and gave their informed consent. The protocol of the study was approved by the ethics committee of the Giannina Gaslini Institute. All the recruited children completed the study protocol.

LUNG FUNCTION AND BRONCHIAL CHALLENGE

All children were able to perform forced expiratory manoeuvres. Forced vital capacity (FVC), FEV_1, and forced expiratory flows at 25–75% of the vital capacity ($FEF_{25-75\%}$) were measured by spirometry (Med Graphics, Pulmonary Function System 1070 Series 2, Med Graphics Corporation; St Paul, Minnesota, USA). On each occasion three forced expiratory manoeuvres were obtained and the best values were retained. All children had baseline FEV_1 of >80% predicted.[10] Aerosols of methacholine were delivered by a dosimeter device (MEFAR, Brescia, Italy) using the same ampoule for each patient.[11] Methacholine solutions were prepared on each study day in 0.9% pyrogen-free saline. The challenge was started with a methacholine dose of 0.02 mg which was then increased in doubling doses until FEV_1, measured within one minute of methacholine inhalation, was less than 80% of the control value (inhalation of saline). The dose of methacholine causing a 20% decrease in FEV_1 (PD_{20} methacholine) was calculated by interpolation of the dose-response curves.[11]

MEASUREMENT OF EXHALED NO

A chemiluminescence analyser (Logan LR 2000 System, Kent, UK) sensitive to NO concentrations from 2 to 5000 parts per billion (ppb) by volume was used. The system was adapted for on-line measurement of NO and therefore did not require exhaled air collection, a potential source of variable loss of reactive NO.[12] Certified NO mixtures (100 ppb) in nitrogen (BOC Gases, Guildford, UK) were used for daily calibration. Environmental NO was measured before and after each study and never exceeded 15 ppb. After flushing the analyser with NO-free compressed air, subjects were asked to perform a slow expiratory vital capacity manoeuvre over 15–20 seconds through a wide bore Teflon tube[12] against a positive pressure of 6–8 cm H_2O. During the manoeuvre oropharyngeal pressure increases sufficiently to cause closure of the soft palate, thereby minimising nasal NO contamination.[12] Expiratory flow was maintained at 150–200 ml/s with the aid of visual feedback. The mean flow rate was 157 ml/s. The concentration of NO in exhaled air was recorded continuously at a rate of 250 ml/min and compared with the signal generated by the calibration mixture.

Typically, the NO concentration peaks early during expiration, probably because of the contribution of nasal NO.[12] This peak is followed by a plateau which is believed to represent NO from the lower respiratory tract.[12] Mean plateau values were calculated for each exhalation. The highest value from three successive reproducible recordings obtained at two minute intervals was retained for statistical analysis. All measurements were made by two independent observers who were unaware of the patient's health status. Exhaled NO levels

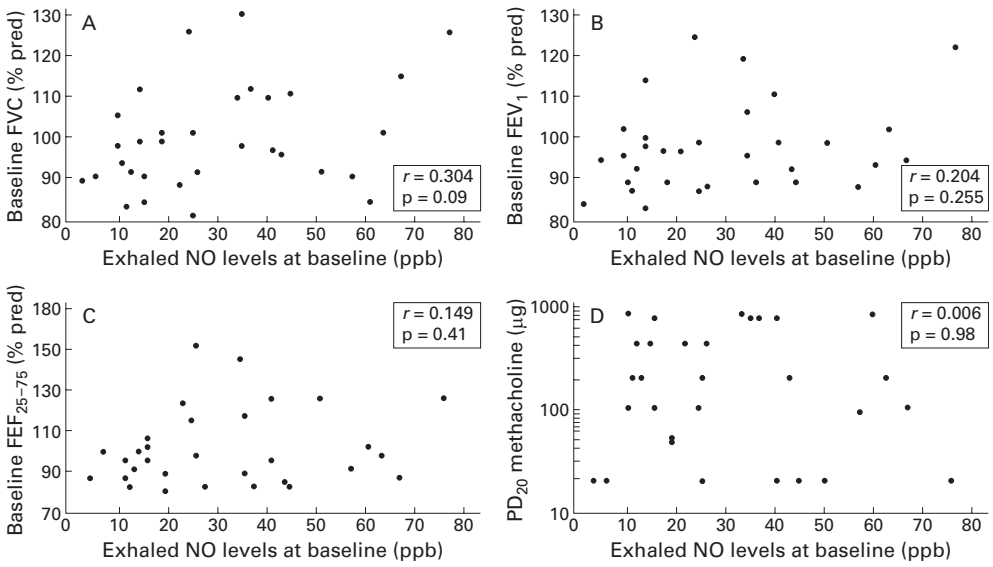

Figure 1 Relationship between nitric oxide (NO) levels in orally exhaled air and (A) FVC, (B) FEV_1, (C) $FEF_{25-75\%}$ at baseline or (D) airway hyperresponsivenss to methacholine (PD_{20} methacholine). None of the correlations was statistically significant using either Pearson's or Spearman's correlation coefficients.

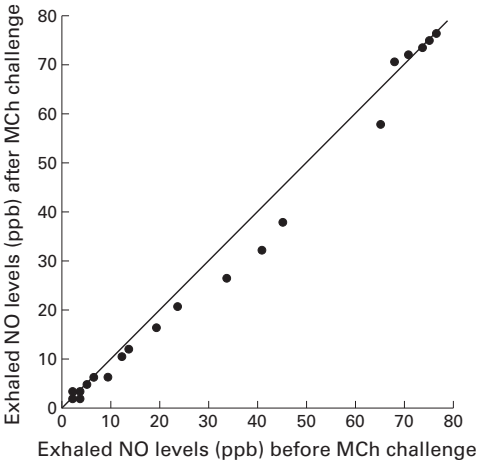

Figure 2 Individual values of exhaled NO (ppb) before and after challenge with methacholine in asthmatic children. There was a significant decrease in NO levels after challenge ($p<0.05$).

were measured at baseline and five minutes after the end of the methacholine challenge.

The repeatability of NO measurements in orally exhaled air was evaluated according to the method of Bland and Altman.[13] For this purpose, two measurements taken on the same day in 10 children were compared. The mean difference in NO measurements of air exhaled from the lungs between the two measurements was 0.38 (0.4) ppb (NS). The coefficient of repeatability was 2.2 ppb.

STATISTICAL ANALYSIS

Age, baseline lung function, and NO levels are presented as mean (SD). PD_{20} methacholine values were log transformed before statistical analysis and are presented as geometric means. The Student's unpaired or paired *t* tests were used when appropriate. Correlations were analysed by Spearman's rank or Pearson's correlation coefficient. The level of statistical significance was set at $p<0.05$.

Results

Baseline FVC, FEV_1, and $FEF_{25–75\%}$ did not differ significantly between the asthmatic and healthy children (table 1). Exhaled NO was detected in all subjects and was significantly higher in the asthmatic children than in the control group (30.0 (19.2) ppb versus 4.1 (2.6) ppb; $p = 0.0001$). Of the 32 asthmatic children, 30 had NO levels above 8.8 ppb (more than 2 SD above the mean of healthy controls).

In the asthma group baseline lung function or airway responsiveness to methacholine did not differ between those children sensitised to one and those sensitised to more than one allergen. No significant correlations were found in these subjects between baseline exhaled NO levels and lung function parameters (FVC, FEV_1, $FEF_{25–75\%}$) or PD_{20} methacholine ($p \geq 0.09$ for each correlation; fig 1). Exhaled NO levels were slightly less after methacholine challenge than before (25.0 (18.0) ppb versus 30.0 (19.2) ppb; $p<0.05$; fig 2).

Discussion

The results of this study show that, in children with mild intermittent asthma, exhaled NO levels are increased compared with healthy controls. However, this increase is independent of the degree of impairment of pulmonary function and of airway hyperresponsiveness.

There is convincing evidence that airway inflammation may be present even in patients with mild intermittent asthma, suggesting that an ongoing recruitment and activation of inflammatory cells may also be present in asymptomatic asthmatic subjects.[11 14 15] Although it is not known whether this "subclinical" inflammation may lead to irreversible airway remodelling, it seems reasonable to evaluate the presence of airway inflammation in asymptomatic patients in order to identify individuals (children and/or adults) who may need a closer follow up and, possibly, anti-inflammatory treatment.[2] The observation that NO levels are increased in the airways of asthmatic subjects has excited considerable interest.[16–18] Indeed, several reports have suggested that measurement of this highly reactive molecule in orally exhaled air may provide a simple non-invasive method of measuring airway inflammation.[6 7 19 20] Even though the relative contribution of the different cellular sources of NO is unknown, a number of observations have suggested that, in asthma, NO originates mainly from the inducible form of nitric oxide synthases (iNOS or type II NOS).[21] This enzyme is induced by proinflammatory cytokines in various cells including macrophages and airway epithelial cells.[21] Similarly, the pathways linking the inflammatory events that characterise asthma with NO production in the airways are not known, but the observation that corticosteroids reduce both exhaled NO and bronchial hyperreactivity[22] has suggested that these two characteristics of bronchial asthma may share some common mechanisms.

A causal relationship between airway inflammation and hyperresponsiveness in allergic asthma has been suggested by a number of studies based mainly on the observation that acute exposure to allergens causes enhanced airway responsiveness and inflammatory cell recruitment in the airways.[14 15 23 24] This concept, however, is challenged by three observations: (1) morphological and functional studies have shown that airway hyperresponsiveness may be sustained by airway remodelling[25] and by the inability to dilate constricted airways[26]; (2) treatment with inhaled corticosteroids does not result in a consistent reduction of airway hyperresponsiveness and airway inflammation[27]; and (3) recent studies have shown frequent dissociation between the numbers of inflammatory cells in the airways and airway hyperresponsiveness in allergic asthma.[8 28 29]

Our study in steroid-naive asthmatic children also failed to show any significant correlation between airway inflammation, as inferred from exhaled NO levels, and bronchial responsiveness to methacholine measured as PD_{20} methacholine. A similar lack of correlation was

also reported by some authors studying steroid-naive adults with mild intermittent asthma[30 31] and steroid-treated adults with unstable asthma.[32] In contrast, Dupont *et al*, in a similar group of steroid-naive adult patients, found highly significant correlations between NO levels in orally exhaled air and bronchial hyperresponsiveness (PD_{20} methacholine) to histamine.[33] Whether these different results are related to patient characteristics or to different mechanisms of action of the two agents used (methacholine and histamine) is unknown.[30 34]

The main statistical issue in this paper is whether there is sufficient power to detect possible relationships between exhaled NO and lung function parameters or bronchial hyperreactivity. However, the observation that the highest correlation reported (between baseline FVC and exhaled NO levels; $r = 0.304$, $p = 0.09$) has a direction (as do all the other relationships) contrary to the implied hypothesis makes the issue of insufficient statistical power unlikely.

After methacholine the levels of exhaled NO were slightly, but significantly, less than at baseline. A similar result was reported by de Gouw *et al* in steroid-naive adults with mild to moderate persistent asthma[35] and also by Garnier *et al*[36] in five of seven subjects investigated. The mechanism underlying the reduction in exhaled NO levels during acutely induced bronchoconstriction is not completely understood, although a back diffusion of NO into the capillary bed at the bronchiolar level could play a role. This could be the result of rapid binding of NO with haemoglobin.[37 38] The hypothesis that decreases in NO after methacholine challenge could be related to reduced airway calibre is not supported by the observation here reported that the decrease in exhaled NO levels did not correlate with changes in lung function parameters.

In conclusion, the results of this study indicate that exhaled NO levels do not seem to be accurate predictors of the degree of airway responsiveness to inhaled methacholine in children with mild intermittent asthma, and confirm the hypothesis that airway inflammation may not be strictly related to bronchial hyperreactivity.[8 25 28 29] This is in line with the current understanding that airway hyperresponsiveness is the result of complex inflammatory and non-inflammatory mechanisms and that biological markers cannot be a surrogate of direct measurements of functional responses to bronchoconstrictor stimuli.[39] Other factors, such as autonomic dysfunction, recent subclinical viral infections, and early changes causing airway remodelling may all be important determinants of the inter-individual variability of airway responsiveness to methacholine in childhood asthma.[25 28 39]

The clinical implications of these results are that single measurements of exhaled NO cannot provide information on airway responsiveness in children with mild intermittent asthma. Further studies are required to establish the relative clinical relevance of exhaled NO, of bronchial hyperreactivity to methacholine or to histamine, and of methacholine or histamine induced bronchoconstriction as indices of disease severity.

This study was supported by Ricerca Corrente from Ministero della Sanità, Rome, Italy and a grant to DS from Valeas SpA, Milan, Italy.

1 Kay AB. Asthma and inflammation. *J Allergy Clin Immunol* 1991;**87**:893–907.
2 National Institutes of Health. *Global initiative for asthma.* NIH Publication No. 95-3659. Bethesda: National Institutes of Health, National Heart, Lung and Blood Institutes, 1995.
3 Galvez RA, McLaughlin FJ, Levison H. The role of the methacholine challenge in children with chronic cough. *J Allergy Clin Immunol* 1987;**79**:331–5.
4 Pizzichini E, Pizzichini MM, Efthimiadis A, *et al*. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid phase measurements. *Am J Respir Crit Care Med* 1996;**154**:308–17.
5 Smith DL, Deshazo RD. Bronchoalveolar lavage in asthma. *Am Rev Respir Dis* 1993;**148**:523–32.
6 Barnes PJ, Liew FY. Nitric oxide and asthmatic inflammation. *Immunol Today* 1995;**16**:128–30.
7 Kharitonov SA, Yates D, Barnes PJ. Inhaled glucocorticoids decrease nitric oxide in exhaled air of asthmatic patients. *Am J Respir Crit Care Med* 1996;**153**:454–7.
8 Crimi E, Spanevello A, Neri M, *et al*. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998;**157**:4–9.
9 Silvestri M, Oddera S, Crimi P, *et al*. Frequency and specific sensitization to inhalant allergens within nuclear families of children with asthma and/or rhinitis. *Ann Allergy Asthma Immunol* 1997;**79**:512–6.
10 Veneruso G, de Benedictis FM, de Martino M, *et al*. Spirometric reference values for an Italian pediatric population. *Riv Ital Pediatr* 1987;**13**:674–81.
11 Oddera S, Silvestri M, Balbo A, *et al*. Airway eposinophilic enflammation, epithelial damage and bronchial hyperresponsiveness in patients with mild-moderate stable asthma. *Allergy, Eur J All Clin Immunol* 1996;**51**:100–7.
12 Kharitonov S, Alving K, Barnes PJ. Exhaled and nasal nitric oxide measurements: recommendations. *Eur Respir J* 1997; **10**:1683–93.
13 Bland JM, Altman DG. Measurement error. *BMJ* 1996;**313**: 744.
14 Bradley BL, Azzawi M, Jacobsen M, *et al*. Eosinophils, T-lymphocytes, mast cells, neutrophils and macrophages in bronchial biopsy specimens from atopic subjects with asthma: Comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991;**88**:661–74.
15 Brusasco V, Crimi E, Gianiorio P, *et al*. Allergen-induced increase in airway responsiveness and inflammation in mild asthma. *J Appl Physiol* 1990;**69**:2209–14.
16 Artlich A, Hagenah JU, Jonas S, *et al*. Exhaled nitric oxide in childhood asthma. *Eur J Pediatr* 1996;**155**:698–701.
17 Dotsch J, Demirakça, Terbrack HG, *et al*. Airway nitric oxide in asthmatic children and patients with cystic fibrosis. *Eur Respir J* 1996;**9**:2537–40.
18 Lundberg JON, Nordvall SL, Weitzberg E, *et al*. Exhaled nitric oxide in paediatric asthma and cystic fibrosis. *Arch Dis Child* 1996;**75**:323–6.
19 Silvestri M, Spallarossa D, Frangova Yourukova V, *et al*. High proportion of atopic children with mild-intermittent asthma has increased orally exhaled nitric oxide levels which are related to the degree of blood eosinophilia. *Eur Respir J* 1999;**13**:321–6.
20 Kharitonov SA, Chung KF, Evans DJ, *et al*. Increased exhaled nitric oxide is asthma is mainly derived from the lower respiratory tract. *Am J Respir Crit Care Med* 1996;**153**:1773–80.
21 Yates DH, Kharitonov SA, Worsdell M, *et al*. Exhaled nitric oxide is decreased after inhalation of a specific inhibitor of inducible nitric oxide synthase in asthmatic but not in normal subjects. *Am J Respir Crit Care Med* 1996;**154**:247–50.
22 Yates DH, Kharitonov SA, Robbins RA, *et al*. Effects of nitric oxide synthase inhibitor and glucocorticosteroid on exhaled nitric oxide. *Am J Respir Crit Care Med* 1995;**152**: 892–6.
23 Rossi GA, Crimi E, Oddera S, *et al*. The late-phase asthmatic reaction to inhaled allergen is associated with an early recruitment of eosinophils in the airways. *Am Rev Respir Dis* 1991;**144**:379–83.
24 Kharitonov SA, O'Connor BJ, Evans DJ, *et al*. Allergen-induced late asthmatic reactions are associated with elevation ofexhaled nitric oxide. *Am J Respir Crit Care Med* 1995;**151**:1894–9.
25 Wiggs BR, Bosken C, Paré PD, *et al*. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1991;**145**:1251–8.
26 Skloot G, Permutt S, Togias AG. Airway hyperresponsiveness in asthma: a problem of limited smooth muscle relaxation with inspiration. *J Clin Invest* 1995;**96**:2393–403.
27 Jeffery PK,Godfrey RW, Adelroth E, *et al*. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. *Am Rev Respir Dis* 1992;**45**:890–9.
28 Haley KJ, Drazen JM. Inflammation and airway function. What you see is not what you get. *Am J Respir Crit Care Med* 1998;**157**:1–4.
29 Brusasco V, Crimi E, Pellegrino E. Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax* 1998;**53**:992–8.
30 Taylor DA, Lim S, Barnes PJ, *et al*. Exhaled nitric oxide production and increased airway responsiveness in asthma

reflects different inflammatory pathways. *Eur Respir J* 1996;**9**:416S.
31 Silkoff PE, McClean PA, Slutsky AS, *et al*. Exhaled nitric oxide and bronchial reactivity during and after inhaled beclomethasone in mild asthma. *J Asthma* 1998;**35**:473–9.
32 Horvàth I, Donnelly LE, Kiss A, *et al*. Combined use of exhaled hydrogen peroxide and nitric oxide in monitoring asthma. *Am J Respir Crit Care Med* 1998;**158**:1042–6.
33 Dupont LJ, Rochette F, Demedts MG, *et al*. Exhaled nitric oxide correlates with airway hyperresponsiveness in steroid-naive patients with mild asthma. *Am J Respir Crit Care Med* 1998;**157**:894–8.
34 Deykin A, Halpern O, Massaro AF, *et al*. Expired nitric oxide after bronchoprovocation and repeated spirometry in patients with asthma. *Am J Respir Crit Care Med* 1998;**157**: 769–75.
35 de Gouw HWFM, Hendriks J, Woltman AM, *et al*. Exhaled ntiric oxide (NO) is reduced shortly after bronchoconstriction to direct and indirect stimuli in asthma. *Am J Respir Crit Care Med* 1998;**58**:315–9.
36 Garnier P, Fajac I, Dessanges JF, *et al*. Exhaled nitric oxide during acute changes of airways calibre in asthma. *Eur Respir J* 1996;**9**:1134–8.
37 Sharma VS, Taylor TG, Gardiner R. Reaction of nitric oxide with haem proteins and model compounds of haemoglobin. *Biochemistry* 1987;**26**:3837–43.
38 Iwamoto J, Pendergast DR, Suzuki H, Krasney JA. Effect of graded exercise on nitric oxide inexpired air in humans. *Respir Physiol* 1994;**97**:333–45.
39 Haley K, Drazen JM. Inflammation and airway function in asthma. What you see is not necessarily what you get. *Am J Respir Crit Care Med* 1998;**157**:1–3.