

Asthma severity and inflammation markers in children

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The relationship of airway inflammation with asthma severity remains unclear. Our aim was to correlate the results of recommended methods of assessment of inflammation with measures of asthma control, in children with a wide range of asthma severity. The study was a cross-sectional investigation of 58 children receiving a wide range of treatment, including 10 treated without regular maintenance therapy and 29 treated with high-dose inhaled corticosteroids (CS). Exhaled nitric oxide (NO), serum eosinophil cationic protein (ECP), and induced sputum (processed for eosinophil count and ECP level) were related to recent symptoms, lung function, and bronchial responsiveness. There was no significant correlation between the results of any method. Neither did any marker of airway inflammation relate to recent symptoms, unlike PC_{20} , which did. There was a significant, inverse correlation between the forced expiratory volume in 1 s (FEV_1) and both NO and sputum ECP ($r=-0.46$, $p<0.001$; $r=-0.48$, $p=0.004$, respectively). Sputum eosinophils were inversely related to the dose of methacholine that corresponded to a 20% fall in FEV_1 (PC_{20}) ($r=-0.57$, $p=0.02$). Serum ECP did not relate to any measure of asthma control. There was no association of any recommended inflammation markers with current symptoms and only a weak relationship between them and physiological measures. The place of these markers remains unclear and their use in clinical practice needs further investigation by long-term longitudinal studies.

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One goal of asthma treatment is to abolish the underlying airway inflammation known to be associated with the condition. A number of non-invasive methods have been recommended for assessing airway inflammation. These include investigation of induced sputum, measurement of exhaled nitric oxide (NO), and the assessment of evidence of systemic eosinophil activation (1–3). In adults, the relationship between these markers is variable. It is therefore not clear which is the most appropriate for use in clinical practice. Furthermore, the relationship between individual markers and clinical severity is controversial, with some researchers finding a correlation with the degree of severity (4,5) while others do not (3,6,7). We hypothesized that in children, symptoms may show a clearer relationship with underlying airway

inflammation because the confounding effects of active smoking and irreversible airway remodeling are probably less marked. Few studies in children have considered a relationship between measures of airway inflammation and asthma severity (3,4,8). The purpose of this study was to assess airway inflammation by using three different recommended methods (induced sputum, exhaled NO and serum eosinophil cationic protein [ECP]) in children with asthma of a wide range of clinical severity, and to relate the findings to current symptom severity. The aim was to determine the best method for use in clinical practice. As the patients at this tertiary referral center tended to have severe asthma, children who no longer attended the center because their symptoms were mild, were contacted and also enrolled.

Patients and methods

Study design

This was a cross-sectional comparison of four different markers of airway inflammation (exhaled NO, sputum eosinophils, sputum ECP, and serum ECP) with measures of asthma control (reported symptoms, lung function, and bronchial responsiveness), in children with a wide range of asthma severity.

Subjects

Children, fulfilling the American Thoracic Society's diagnostic criteria of asthma, were enrolled into the study if they fell into one of the following categories:

1 The child was undergoing investigation for asthma, which was difficult to control despite treatment with doses of inhaled steroids of at least 1 mg/day ($n=24$).

2 Random selection from the asthma clinic ($n=19$).

3 Children with mild asthma, no longer attending the clinic, were obtained from outpatient records ($n=15$).

These three categories were chosen to ensure incorporation of a wide range of symptom severity.

Fifty-eight children (6–19 years of age; 62% male) were enrolled (Table 1). Ten were treated with bronchodilators only, 11 were taking low-dose inhaled corticosteroids (CS) ($<600 \mu\text{g/day}$), 29 high-dose inhaled CS ($\geq 800 \mu\text{g/day}$), and eight oral CS, either on alternate days or daily (Table 1). Eighteen children took long-acting bronchodilators, but not on the day of study, and 10 took them on the study day as they were not able to manage without.

Asthma severity

Asthma severity was determined as described below.

1 Maintenance medication category:

- no CS,
- inhaled CS up to $600 \mu\text{g/day}$,
- inhaled CS at least $800 \mu\text{g/day}$, or
- inhaled CS at least $800 \mu\text{g/day}$ +oral CS.

2 Functional severity, i.e. symptoms despite preventative treatment (9), defined as the average reported symptom frequency per week in the previous 4 weeks, sufficient to require rescue bronchodilator:

- none,
- fewer than three times/week, or
- at least three times per week.

Protocol

The children attended the asthma laboratory on one occasion. Short-acting bronchodilators were stopped for at least 8 h and, when possible, long-acting ones for at least 12 h. If this was not possible for clinical reasons, this was recorded and the children were tested having taken them, but they omitted the methacholine challenge.

The following investigations were carried out:

1 A questionnaire on symptoms and rescue medication use in the past month.

2 Baseline spirometry that was recorded from the best of three attempts, of values that were within 5% of each other, using a dry wedge spirometer (Vitalograph compact, Maids Moreton, Bucks., UK).

3 Measurement of exhaled NO by using a chemiluminescence analyzer (Model LR2000; Logan Research, Rochester, Kent, UK), which had a sensitivity of 1 p.p.b. to 100 p.p.m. and an accuracy of ± 0.5 p.p.b. The measurements were carried out according to the European Respiratory Society (ERS) guidelines (10). The child took a maximal inspiration then exhaled slowly for ≈ 15 –20 s or until the NO trace showed a definite plateau. The expiratory flow rate and pressure were kept within the desired range of 5–6 l/min and 3 ± 0.4 mmHg, respectively, by the use of visual and auditory aids. The mean of three acceptable attempts (obvious plateau and optimal expiratory flow or pressure) was recorded.

4 Methacholine challenge was performed using a dosimeter (11) (Mephar, Brescia, Italy) if the forced expiratory volume in 1 s (FEV_1) was at least 65% predicted and no long- or short-acting bronchodilator had been taken that day. Methacholine was inhaled in doubling doses (0.01–16 g/l). Lung function was measured at 90 and 120 s after each inhalation and the inhalations were continued until at least a 20% fall in FEV_1 was obtained or the maximal dose reached. The dose of methacholine that corresponded to a 20% fall in FEV_1 (PC_{20}) was calculated by linear extrapolation from the log dose–response curve.

5 Following the methacholine challenge, nebulized salbutamol (2.5 mg) was inhaled. Sputum induction was attempted if the FEV_1 after salbutamol was at least 65% predicted. The child rinsed the mouth, to minimize salivary contamination, before inhaling ultrasonically nebulized hypertonic saline (3.5%) aerosol for 5-min periods, between which the child was encouraged to expectorate and the lung function was checked. The inhalation was terminated if the FEV_1 fell by 20%, otherwise it was continued

Table 1. Subject characteristics according to medication category

Medication category (ICS $\mu\text{g/day}$)	n	Age (yr) Median (range)	FEV_1 (%) Mean (SD)	PC_{20} G. mean
None	10	13 (9–19)	90 (15)	1.71
$\leq 600 \mu\text{g}$	11	10 (7–15)	85 (15)	0.62
$\geq 800 \mu\text{g}$	29	12 (6–17)	82 (12)	0.96
$\geq 800 \mu\text{g} + \text{oral CS}$	8	13 (8–19)	74 (15)	1.09
Total	58	12 (6–19)	82 (14)	0.95

CS, corticosteroid; FEV_1 , forced expiratory volume in 1 s; G. mean, geometric mean; ICS, inhaled corticosteroid; PC_{20} , the dose of methacholine that corresponds to a 20% fall in FEV_1 .

until a sputum sample was obtained or a total inhalation time of 20 min had been achieved.

6 After application of a local anesthetic cream, a blood sample was obtained for analysis of total and specific immunoglobulin E (IgE) (to house dust mite, cat, grass and dog, if one was present at home) and serum ECP. For the latter, blood was collected in plain glass tubes and allowed to clot at room temperature for 1–2 h before separation of the serum, which was frozen at -20°C for subsequent analysis by radioimmunoassay (RIA) (Pharmacia Diagnostics, Uppsala, Sweden) (12).

Sputum processing

The sputum sample was processed within 30 min if left at room temperature or refrigerated for no longer than 1 h. One millilitre of Hank's balanced salt solution (HBSS) containing 1% dithiothreitol (DTT) (Sigma Chemical Co., Poole, Dorset, UK) was added to the sputum before inverting repeatedly to homogenize the sample. The sputum volume was measured and made up to 5 ml with HBSS. After mixing, the preparation was centrifuged at 400 g for 10 min at 4°C . The resulting cell pellets were resuspended in 1 ml of HBSS for evaluation of total cell counts using a hemocytometer and Kimura staining. Slides were prepared using a cytospin instrument (Shandon, Runcorn, Cheshire, UK) and were stained using May–Grunwald–Giemsa stain for differential cell counting. A cytospin preparation containing at least 400 non-squamous cells with less than 65% squamous cells, was considered a satisfactory sample.

Presence or absence of inflammation

Inflammation was considered to be present if at least one of the indicators of airway inflammation was outside the range we have previously found in normal children ($\text{NO} > 12.5$ p.p.b. (13); sputum eosinophils $> 1\%$; sputum ECP > 44 IU/l). Serum ECP was not included as it did not correlate with any index of asthma control. Children without any sputum results were excluded unless the NO level was raised, as a raised NO level was one of our indicators of airway inflammation.

Statistical analysis

Statistical analysis was performed using Minitab for Windows, version 12 (Minitab Ltd. State College, PA, USA) which allows for missing data. Results of PC_{20} , serum ECP and IgE, sputum ECP and sputum eosinophil differential count, were log transformed in order to normalize data distribution. Comparisons between groups were made using a one-way analysis of variance or a non-paired *t*-test. Significant associations between results were sought by using a Pearson product moment correlation coefficient with log-transformed data, where appropriate. Where no response to methacholine was obtained at the highest concentration given (16 g/l), a value of 32 g/l was used in statistical calculations.

Results

Methacholine challenge was measured in 36 children. Eighteen of these children reported symptoms occurring fewer than three times per week (severity groups 1 and 2) and 18 at least three times per week (group 3). PC_{20} was significantly lower in the latter group (mean log $PC_{20} \pm \text{SD}$: 0.21 ± 0.8 vs. 0.3 ± 0.4 , $p = 0.01$), but there was no significant difference in FEV_1 (%) (mean $\pm \text{SD}$: 84 ± 12 vs. 81 ± 14).

Results for serum total IgE were available in 44 children and for house dust mite radioallergo-sorbent test (RAST) in 50. RAST to house dust mite was significantly greater in those reporting fewer than three symptoms per week than in those with more frequent symptoms (mean log $\text{RAST} \pm \text{SD}$: 1.63 ± 0.77 vs. 0.96 ± 0.90 , $p = 0.01$).

Relation of indices of inflammation markers to asthma severity

Exhaled NO. Satisfactory recordings of exhaled NO were obtained in all children. NO values showed a significant, inverse correlation with baseline FEV_1 , both including and excluding the 10 children who had taken a long-acting bronchodilator that day ($r = -0.46$, $p = 0.001$; $r = -0.45$, $p = 0.001$, respectively, Fig. 1). NO showed no

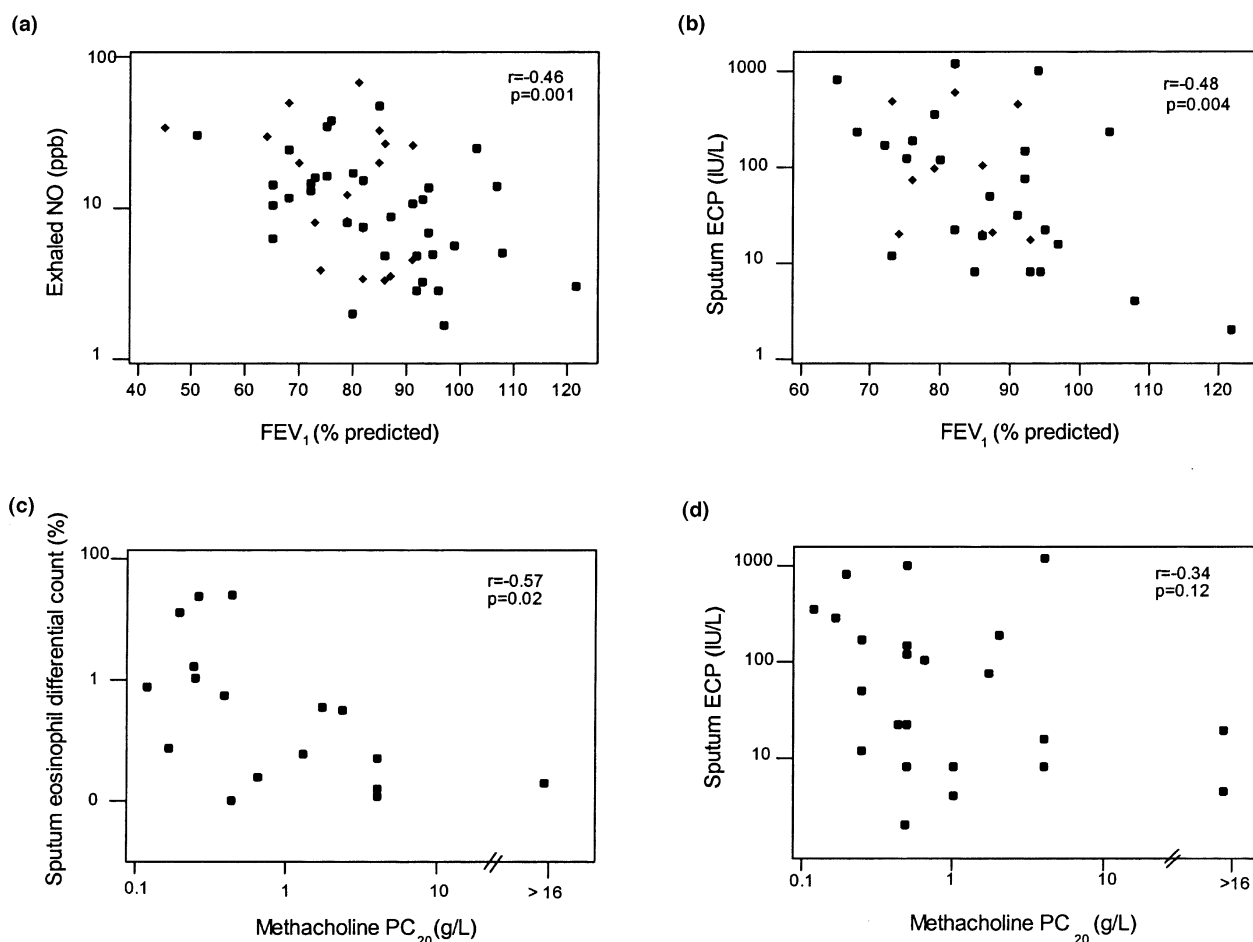


Fig. 1. Correlation of: (a) the forced expiratory volume in 1 s (FEV_1) and nitric oxide (NO); (b) sputum eosinophil cationic protein (ECP) and FEV_1 ; (c) sputum eosinophils and methacholine PC_{20} (the dose of methacholine that corresponded to a 20% fall in FEV_1); and (d) sputum ECP and methacholine PC_{20} . ■, no long-acting bronchodilator taken; ♦, long-acting bronchodilator taken on the day of the study.

relationship with recently reported symptoms ($p=0.83$, Fig. 2) or with treatment category. The correlation between NO and PC_{20} was not significant ($r=-0.21$, $p=0.3$). NO was significantly associated with total serum IgE and with RAST to house dust mite ($r=0.37$, $p=0.02$; $r=0.52$, $p<0.001$; respectively).

Induced sputum. Satisfactory sputum samples were obtained in 36 children (cell counts were not possible in six of these children for technical reasons, such as cell clumping or air bubbles). In 21 children, both PC_{20} and sputum ECP results were available and, in 17 children, PC_{20} and sputum eosinophils. There was an inverse correlation between baseline FEV_1 and sputum ECP ($r=-0.48$, $p=0.004$, Fig. 1) but not with the proportion of eosinophils ($r=-0.33$, $p=0.1$). For PC_{20} , there was a significant correlation with the proportion of eosinophils ($r=-0.57$, $p=0.008$) but not with ECP ($r=-0.34$, $p=0.12$) (Fig. 1). There was no significant difference in either

between different symptom groups (Fig. 2) or treatment category (Fig. 3).

Serum ECP. Data on serum ECP was available in 40 children. There was no relationship between serum ECP and FEV_1 ($r=-0.08$, $p=0.6$) or PC_{20} ($r=0.18$, $p=0.39$), nor were there any differences in serum ECP between severity of asthma (Fig. 2) or treatment groups ($p>0.1$).

Interrelation between inflammation markers

There were no significant correlations among sputum eosinophils, sputum ECP, serum ECP, and NO (Table 2).

Presence or absence of inflammation

Results were available on the presence or absence of inflammation in 51 children. Evidence of inflammation was present in 40 (78%), in whom both FEV_1 (mean $\pm$ SE: 90 ± 3.2 vs. 80 ± 2.1 , $p=0.02$) and PC_{20} (log mean $\pm$ SE: 0.50 ± 0.29 vs. -0.20 ± 0.1 ,

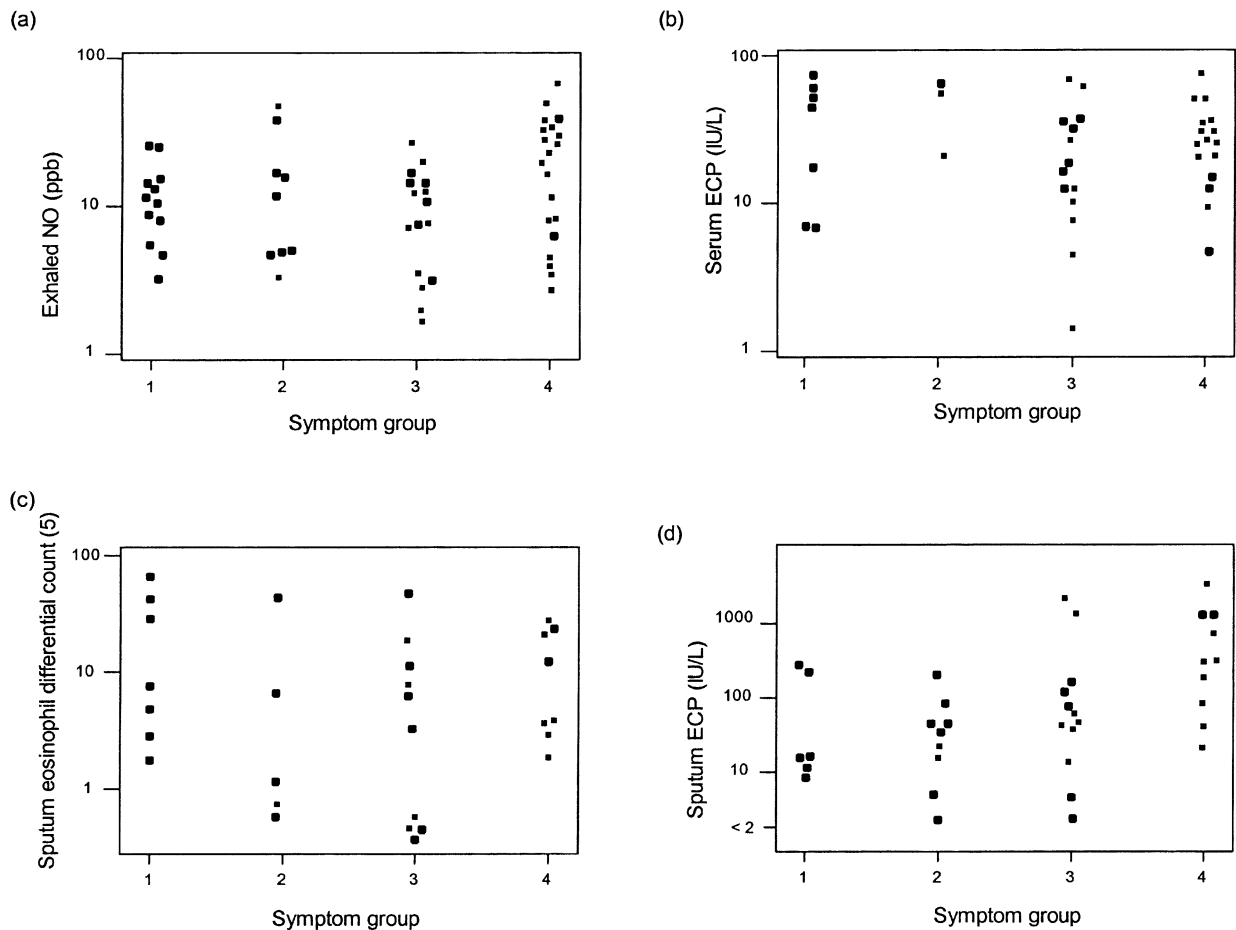


Fig. 2. (a) Nitric oxide (NO); (b) serum eosinophil cationic protein (ECP); (c) sputum eosinophils (%); and (d) sputum ECP, according to symptoms over the preceding 4 weeks: 1, none; 2, less than three symptoms per week; 3, more than three symptoms per week; 4, 'difficult asthma'. Smaller squares, long-acting bronchodilator taken during the assessment period.

$p = 0.04$) were significantly reduced. All those with severe asthma had some evidence of inflammation (18/18), but so did 75% (nine of 12) of children reporting no symptoms in the preceding month.

Discussion

This cross-sectional study of children with a wide range of asthma severity has shown that a number of potential markers of airway inflammation failed to correlate with symptoms reported in the preceding 4 weeks. When we simplified the analysis as to whether there was or was not any evidence of inflammation present, all those who

were included because of difficult-to-control asthma showed some evidence of inflammation; however, so did 75% of those reporting no recent symptoms. In contrast, methacholine responsiveness was significantly increased in those with recent symptoms. We also showed that the inflammation markers used, apart from serum ECP, correlated with some physiological measure associated with asthma control, namely lung function and/or bronchial responsiveness. However, we found no interrelation between the different markers.

The methods used in this study have all been recommended for monitoring inflammation in

Table 2. Inter-correlation between inflammatory markers*

	NO	SE (%)	Sputum ECP	Serum ECP
NO	—	−0.01	0.09	0.15
SE (%)	—	—	0.23	0.29
Sputum ECP	—	—	—	0.94

*The results were determined by using the Pearson correlation coefficient with log-transformed data. SE, sputum eosinophils.

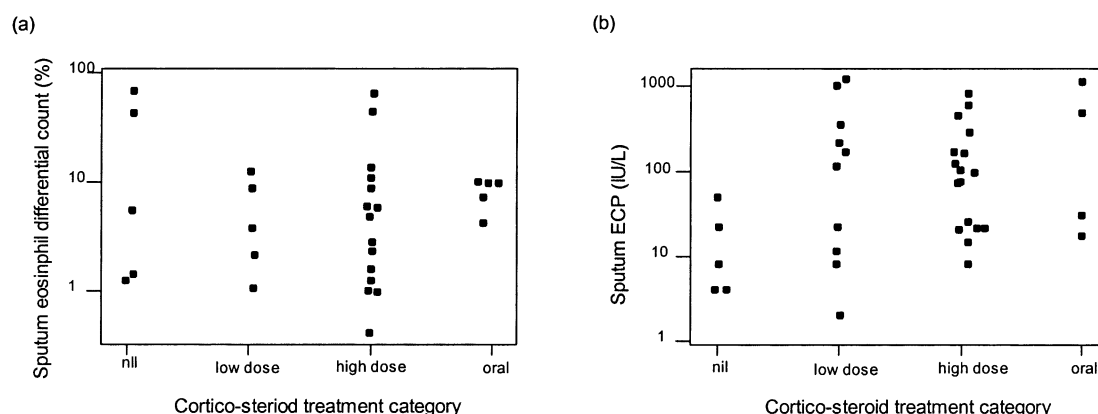


Fig. 3. (a) Sputum eosinophil (%) and (b) sputum eosinophil cationic protein (ECP), according to corticosteroid (CS) treatment category.

children in a clinical setting and have acceptable reproducibility (2,3,14). There is, however, a large step between using an inflammation marker to differentiate asthma from non-asthma, and its use to monitor treatment.

We had postulated that the relationship between symptoms and evidence of airway inflammation might be clearer in children than in adults, but this was not so. One explanation could be the confounding effect on reported symptoms of long-acting bronchodilators that were used by many of the subjects. This is unlikely to be the case, as despite taking them, most of the children had frequent symptoms (Fig. 2). Currently, there is no recognized method of defining asthma symptom severity. Clinical categorization by symptoms occurring fewer than three or at least three times per week, has some clinical relevance as this is often used as a possible indication for an increase in anti-inflammatory therapy. The implicit assumption is that on-going inflammation is responsible for continuing symptoms. Our results do not support this. Indeed, even when direct evidence of eosinophilic inflammation has been sought from bronchial biopsies – the gold standard – poor correlation has been found between mucosal inflammation and asthma control (15). It is not clear whether the measures of clinical control are inadequate, or more probably, symptoms are a complex integration of a number of factors (16), some of which are independent of eosinophilic inflammation.

Another possibility is that the number of participants in the study was too small to demonstrate such a relationship, although it was similar in size to some others in children (8,14), and for markers of inflammation to be of clinical value they must be applicable to single individuals. A sputum sample was only obtained from a proportion of children, owing either to poor lung function or to a lack of sputum

production. However, a type two error is unlikely to be the explanation of our findings. For the small differences that were found in either sputum eosinophils or ECP, we would have required to study ≈ 8000 children to detect a significant difference between those reporting symptoms fewer than three times a week compared to those reporting symptoms at least 3 times a week, with 80% power at the 5% level. Changes requiring a study of such size are highly unlikely to be clinically useful or relevant.

Like most studies investigating the correlation between symptoms and inflammation markers, the study was cross-sectional in design. From what we now know about the variability of this relationship between individuals, this may not be the most informative method. Longitudinal data are required to clarify whether any of these measurements have any bearing on long-term outcome.

Our findings are compatible with those reported in adults and the few studies performed in children. In some studies, asthma control has been found to relate to an inflammation marker in steroid-naïve (17,18) but not steroid-treated (5,15,19) subjects. Many studies in adults (5,18,20,21), but not all (20), have shown a relationship between NO or sputum inflammation indices and lung function. In adolescents with severe asthma, evidence of airway inflammation was associated with reduced lung function (8), as it was in our group, which included those with severe disease and many who required high-dose steroid treatment. In contrast, no relationship was found between lung function and NO in children untreated with steroids or in 6–13-year-olds with mild-to-moderate symptoms (14,22). These differences may be a result of the preservation of lung function in those with milder disease of shorter duration. In the current study, we also found methacholine PC_{20} to be

related to sputum eosinophils, but not to sputum ECP and NO. There is inconsistency in the reported relationship between airway inflammation and bronchial responsiveness in adults (21,23,24). This probably reflects the multifactorial nature of increased responsiveness (25), components of which may vary between individuals, and therefore the results are strongly influenced by subject selection.

Like some others (20,26) we found no correlation between the different methods. This could have been because of a varying effect on each marker by CS treatment. The clearest inter-relationship between markers has been seen in steroid-naïve subjects (17,18). Piacentini and colleagues found a correlation between sputum eosinophils with both sputum ECP and NO in steroid-naïve, but not in steroid-treated, children (14). Furthermore, a difference has been found in the response of NO and sputum eosinophils to CS treatment (17,26). Therefore, the different tests probably reflect differing aspects of the inflammatory process, in a manner not yet understood. The end product could be reflected by the level of bronchial responsiveness, as this was increased in children reporting recent symptoms. Interestingly, Sont and colleagues found that bronchial responsiveness correlated with asthma severity, but more direct indices of airway inflammation did not (15).

The paradox of asthma is that, whereas guidelines emphasize the importance of inflammation control and stipulate that anti-inflammatory medication should be given early, inflammation itself is not monitored clinically. This has given rise to studies such as ours, which have attempted to define the relationship between asthma control and evidence of inflammation. The first problem is to identify which method, or methods, of assessment to use. Sputum samples can only be obtained in $\approx 60\%$ of children, but measurement of exhaled NO is almost universally possible in children older than 7 years of age. However, the results of this and other studies have shown little or no correlation between markers in subjects on anti-inflammatory therapy (20,26). The second dilemma is to know how to interpret the results in a clinical context. Indeed, it might be legitimate to ask whether it is worth measuring inflammation at all. Certainly, its measurement has greatly contributed to our understanding of the underlying mechanisms of asthma. This, as our results indicate, are as complex in children as in adults, with no clear relationship between indices of inflammation and symptoms, and

only a loose association between inflammation and functional parameters, such as lung function and bronchial responsiveness. Inflammation may modify the perception of symptoms in some subjects (27). Previous inflammation, resulting in airway remodeling, may be responsible for current symptoms in others (25). On the other hand, some subjects, who are symptom-free, have obvious evidence of inflammation, and others with current symptoms have none.

Many questions remain to be answered. When, considering the very variable course of childhood asthma (28), does untreated (asymptomatic) airway inflammation in children invariably result in long-term adverse sequelae, or does it only occur in some? Should symptomatic subjects, without evidence of inflammation, have further anti-inflammatory treatment? Which method is most appropriate for measuring inflammation? Can some aspects of inflammation, for example a raised NO, ever be protective? Is long-term outcome improved by treating inflammation, as has been shown by guiding treatment by using a measure of bronchial responsiveness as a surrogate for inflammation (29)? Until we know the answers to these questions we will not know how to interpret our attempts to monitor airway inflammation in our patients. The answers will only become apparent from longitudinal studies, performed in well-characterized subjects, considering long-term outcomes. For the benefit of monitoring inflammation in childhood asthma to be evident, there is need for a comparison of treatment regimes that are guided by indices of inflammation or lung function or symptoms, or a combination of all three, throughout childhood. This type of information is particularly important in children, in whom troublesome symptoms persisting into adulthood are seen in only a minority (28) and in whom the side-effects of over-enthusiastic treatment with inhaled CS may be especially relevant.

In summary, using four recommended markers of airway inflammation in a group of children with a wide spectrum of asthma severity, we found no correlation between the methods and no relationship between any method and current symptoms. We did find some association with sputum markers and NO to lung function and/or bronchial responsiveness. It is possible that each method may be giving different, but complementary, information about different aspects of the airway process that leads to asthma. The significance of these findings to clinical practice needs further investigation.

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