

Exhaled NO and eosinophil markers in blood, nasal lavage and sputum in children with asthma after withdrawal of budesonide

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Background: There is a need for controlled trials among children with asthma to evaluate and compare different markers of inflammation.

Objective: Our goal was to investigate the effect of withdrawal of inhaled budesonide on repeated measurements of exhaled NO (ENO), peripheral blood eosinophils (PBE), sputum/NAL/serum-eosinophil cationic protein (ECP), bronchial hyperresponsiveness (BHR) and forced expiratory volume in 1 s (FEV₁) in children with allergic asthma.

Methods: Eighteen asthmatic children were randomly allocated to continue or discontinue use of inhaled budesonide. They were followed up, at six visits for 4 months with regular blood, serum, sputum, and NAL samples. Sixteen age-matched healthy children served as controls. **Results:** ENO, PBE, and S-ECP increased significantly in the withdrawal group ($p < 0.05$) but not in the continuous treatment group. No trend could be observed during the study for markers in sputum or in NAL in either group.

Conclusion: The present data provide evidence for the clinical usefulness of measuring ENO, PBE, and S-ECP and when combined they could help to avoid over- and undertreatment with corticosteroids in the growing child.

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Markers of airway inflammation are needed for prediction of asthma deterioration and evaluation of disease severity. This is particularly important in pediatric care to avoid over- and undertreatment with corticosteroids in the growing child.

However, the accuracy and applicability of possible markers must be carefully evaluated before they can be recommended in daily practice (1).

Several studies have shown that levels of exhaled nitric oxide (ENO) are elevated during inflammatory conditions such as asthma compared with ENO in healthy individuals (2–5). In addition, there is evidence that inhaled corticosteroids can rapidly depress elevated ENO levels back to normal (6, 7). How this decrease relates to symptoms is less certain. ENO can be normal

or elevated during ongoing steroid treatment in patients with symptomatic asthma (8, 9). The non-invasive nature of its measurements, together with the immediate availability of the results, makes ENO ideally suited for monitoring airway inflammation in childhood asthma. The sensitivity and specificity of ENO as a marker of airway disease, needs to be further evaluated, using standardized procedures in different age groups (2–5, 10, 11).

Induced sputum could serve as a direct and more invasive way of measuring airway inflammation. Asthmatic patients frequently have increased eosinophil count and eosinophil cationic protein (ECP) in sputum (sp-ECP) (12), when compared with normal subjects, and it is thought to reflect the severity of airway inflammation in asthmatic patients (13–15). These

elevated levels are sometimes reduced after corticosteroid treatment (12).

The upper airway, specifically the nasal epithelium, shares many properties with the lower airway. Moreover, it is easily accessible and nasal lavage (NAL) procedures are well tolerated even by children (16–18). Various NAL techniques have been presented and suggested to mirror airway inflammation but the main problem is still unsolved, namely how to interpret lavage data, most often of unknown dilution, in a clinical setting (19).

Markers of inflammation in the peripheral blood have been extensively studied. Previous observations have shown that high peripheral blood eosinophil (PBE) count indicates ongoing inflammation and an increased risk of relapse in acute asthma (20). Different eosinophil proteins, i.e. ECP and eosinophil protein X (EPX) have been suggested as markers of eosinophil activity (20–22).

Little information is currently available from adequately controlled trials concerning the impact of withdrawal of steroid treatment on airway inflammation as reflected in blood, sputum, NAL, and exhaled air.

The aim of this placebo-controlled corticosteroid withdrawal study was to evaluate the clinical usefulness of different markers of airway inflammation in children with pet allergy and asthma, by investigating levels of ENO, PBE, serum-ECP (S-ECP), sp-ECP, and NAL-ECP (N-ECP) in relation to bronchial hyperreactivity (BHR) and lung function.

Methods

Study design

The study was performed during the pollen season from October to the beginning of April and consisted of a run-in period of 2–6 wk and an intervention period of 4 months. The children were included at the run-in visit and randomized at visit 1 in a double-blind and parallel manner either to continue on budesonide (the continuous treatment group) or to receive placebo (the withdrawal group). They came for six visits during 15 wk. They were allowed to take one oral dose (4 mg) of betamethasone as rescue medicine if worsening of asthma occurred. They were withdrawn from the study if they developed daily symptoms of asthma during normal activity, a need for β_2 agonist more than four times a day, an irreversible 20% decrease in FEV₁ and/or more than 20% decrease in PEF for more than 3 days. The children were assessed at each visit,

between 07.00 and 09.00 AM. At every visit blood, NAL fluid and exhaled gas were obtained and physical examination and lung function tests were performed. Methacholine challenge was performed at the run-in visit, and 5, 9, and 15 wk after randomization. Sputum induction was performed at visits 1 and again after 13 wk.

Subjects

The inclusion criteria were age between 9 and 16 yr, a history of mild to moderate asthma and of allergy to cat and/or dog confirmed by a positive skin prick test (SPT), daily use of budesonide 200 or 400 μ g for more than 3 months and FEV₁ over 80% of predicted. The children were excluded if they were sensitized to house dust mite or had a pet that they were sensitized to. Randomization was stratified for different doses of budesonide (400 μ g a day or 200 μ g a day).

Healthy controls subjects

Sixteen age-matched, healthy children served as controls (Table 1) and were examined once. To exclude atopy, SPT for cat, dog, horse, house-dust mite, molds, birch, grass and mugwort was performed. Blood, NAL fluid and exhaled gas were obtained and methacholine challenge, physical examination and lung function tests were performed.

Skin prick test

SPT was performed with extracts of cat, dog, horse, rat and rabbit (Soluprick 10.000 SQ; ALK, Hørsholm, Denmark) in duplicate in the asthmatic children. A mean wheal diameter of > 3 mm was considered as positive.

NO measurement in exhaled gas

Concentrations of exhaled NO (ENO) were measured using a chemiluminescence analyzer

Table 1. Characteristics of patients and controls at baseline

	Continuous treatment group	Withdrawal group	Healthy controls
n	10	8	16
Mean-age (yr)	10.7	12.2	13.3
Females/males	3/5	7/3	10/6
Budesonide 400 μ g/200 μ g	4/4	7/3	0
Pos. SPT cat	10	8	0
Pos. SPT dog	9	7	0

(Model 270B; Sievers, Boulder, CO, USA) connected to a vacuum pump (model RV5; Edwards, Crawley West, Sussex, UK). In this type of analyzer NO is mixed with ozone upon which NO is oxidized to form the unstable reaction product NO₂ (in an excited state). When NO₂ returns from the excited to the ground state, a photon is emitted and registered by a photomultiplier tube. In theory therefore the number of photons registered should equal the number of NO molecules entering the analyzer. This method has a sensitivity for NO of < 2 pmol/ml of air. A pressure of 6 bar was set for the reaction chamber. The analyzer was calibrated with an NO gas certified to 90 p.p.m. (Air Liquide, Stockholm, Sweden) and a zero filter (Sievers) for zero calibration.

A mouthpiece, pneumotachygraph, and flow resistance was combined to form a fairly small exhalation unit (Fig. 1) that was handheld by the subjects. The sampling tube of the exhalation unit was connected to the analyzer and the pneumotachygraph (Fleisch Model 0; Metabo, Epalinges, Switzerland) was connected to a pressure transducer (Model 4500; Vacumed, Ventura, CA, USA), which in turn was connected to a demodulator card (Model 4512, Vacumed).

The analog signal from the NO analyzer, as well as the flow signal from the demodulator card was digitized at 10 Hz (A/D converter ADC-100; Pico Instruments, Cambridgeshire, UK) and displayed on a computer monitor using an application developed in LabView (Ver. 4.1; National Instruments, Austin, TX, USA). This system enabled the subject to coordinate exhalation effort to comply with the target flow of 4 l/min, as well as helping the operator to identify any leaks or other malfunction during testing. In most cases five exhalations were carried out (but at least four) and the mean of the visually determined plateau levels in the different exhalations was used.

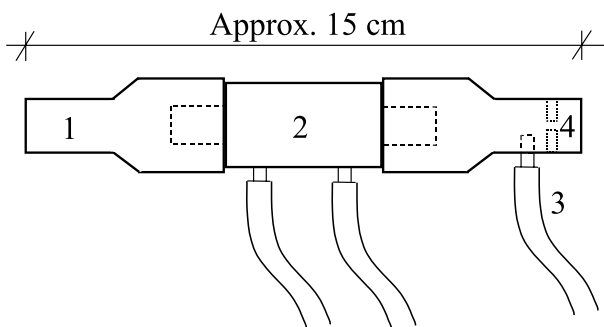


Fig. 1. 1. Mouthpiece, 2. Pneumotachygraph (Fleisch Model 0, see text), 3. Sampling duct to analyzer, 4. Resistance.

Metacholine challenge

Bronchial challenge was performed according to the protocol of Chai et al. (23), with slight modification, with increasing doses of methacholine chloride delivered, using a dosimeter (Spira Elektro 2 nebulizer system; Hamenlinna, Finland). A cumulative dose of minimum 22 µg and maximum 2800 µg was delivered. Forced expiratory manoeuvres were performed 5 min after the start of each inhalation and the challenge was stopped when a greater than 15% fall of FEV₁ occurred or the maximum dose was reached. The bronchial reactivity to methacholine (PD15, provocative dose that causes a 15% fall in FEV₁) was determined from the log dose-response curve by linear interpolation.

Spirometry

Lung function was evaluated by means of flow-volume curves (Vitalograph, Buckingham, UK). The vitalograph was calibrated daily and the values were obtained according to ATS standards.

Sputum induction

Sputum was induced by inhalation of nebulized hypertonic saline in concentrations of 3% and 5%, for 10 min each, until a sputum sample had been obtained (24, 25). The sample was examined within 2 h. Salivary contamination was removed macroscopically from the specimen. The selected portion was weighed and treated with fresh dithiothreitol (DTT) in a balanced salt solution (Sputalysin 10%; Calbiochem, San Diego, CA, USA), diluted to 1% by the addition of distilled water. The selected portion was mixed with an equal volume of the DTT solution. The mixture was vortexed and placed in a 37°C shaking water-bath. After 15 min in the water-bath, the mixture was again vortexed for 5 s and gently aspirated in and out of a pipette to ensure mixing and strained through a nylon gauze. The mixture was then centrifuged for 10 min at 530 g. Spirometry was performed before and after 3% saline inhalation, and after 5% saline inhalation.

NAL

Nasal lavage was performed with the patient sitting and leaning forward (26). This position prevented fluid from reaching the throat. One nasal cavity was lavaged with 6 ml lukewarm 0.9% saline solution with a syringe (10 ml), to which a nasal cone was adapted. To avoid

leakage of fluid, the cone was gently applied to the external part of the nostril. The saline solution was injected into the nasal cavity, left there for 1 min and then aspirated back into the syringe. Thereafter, the nasal wash was kept on ice and centrifuged at 1800 rpm, at +4°C, for 10 min.

Peripheral blood leukocytes

Peripheral blood was collected in tubes containing EDTA (Vacutainer; Terumo, Leuven, Belgium, 5 ml, with 50 µl of 21% EDTA). The absolute number of leukocytes and the proportion of eosinophils were counted.

ECP in serum, NAL and sputum

Sera and the supernatant portion of sputa and NAL were handled according to the suppliers of the ECP-kit (Pharmacia AB, Uppsala, Sweden). ECP was analyzed using a commercially available fluoroimmunoassay (Pharmacia ECP CAP System FEIA; Pharmacia AB, Uppsala, Sweden). The detection limit was 2 µg/l.

Statistical methods

To evaluate the individual changes during the study period, baseline values for each subject were subtracted from the corresponding value at the following visits. Comparison between the groups at separate visits was performed with the Mann–Whitney *U*-test and analysis of variance by ANOVA. Multiple linear regression was used to assess changes in parameters through the study period. Changes from baseline within separate groups were calculated with Wilcoxon's paired rank test. Relationships between variables were evaluated by the Spearman rank correlation coefficient. Results are presented as medians (interquartile ranges).

Informed consent

The study was approved by the local Ethics Committee. Informed consent was obtained from both children and parents.

Results

Subjects

Eighteen children were included in the study. Nineteen were originally recruited but one girl got a severe infection on the day of randomization and was therefore not included in the study.

The remaining 18 children (Table 1) participated according to the study protocol (Fig. 1). Eight children were randomized to the continuous treatment group and 10 children to the withdrawal group (Table 1). Three children allocated to the withdrawal group dropped out because of exacerbation of asthma symptoms, whereas none dropped out in the continuous treatment group. The remaining children attended all visits and participated in all required measurements and 16 healthy children served as controls.

ENO

The repeatability in the NO measurements was good; when the coefficient of variation was calculated for each subject and day, the mean value for the entire study was 4.0% for the exhalation flow rate and 9.6% for NO level.

In the group where budesonide was withdrawn, the NO levels increased from 18–32 p.p.b. (mean) and 13–18 p.p.b. (median), whereas they remained unchanged at 21–22 p.p.b. (mean) and 15–13 p.p.b. (median) in the continuous treatment group. Levels of ENO within the withdrawal group had increased 5 wk after cessation of budesonide ($p = 0.01$) and stayed increased at all visits throughout the study period of 4 months ($p \leq 0.01$). The statistical difference between the withdrawal and the continuous group was significant after 1, 5 and 15 wk (Fig. 2). ENO levels within the continuous treatment group remained unchanged at all visits. Only one child in the budesonide had increasing ENO after 3 months while 5 of 10 in the withdrawal group had increasing ENO after 3 months.

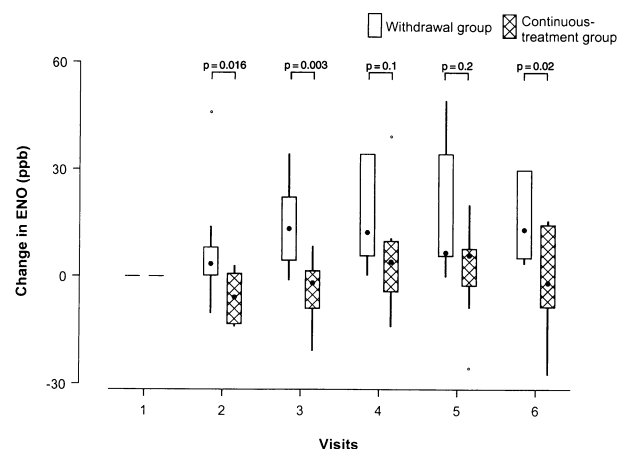


Fig. 2. Change in ENO levels compared with baseline (V_1). The change from baseline values and differences between the groups are presented as median, interquartile range, minimum/maximum and outliers (dots).

At the start of this study no significant difference was seen in the levels of ENO between the budesonide-treated children and healthy children. Five weeks after cessation of budesonide, levels of ENO became significantly higher compared with healthy controls in the withdrawal group, and then stayed higher throughout the study ($p < 0.05$). Only on one of the days when measurements were performed was the background level of NO in ambient air above 5 p.p.b.

Lung function

FEV₁ decreased in the withdrawal group and the difference to the continuous treatment group was significant when all data were pooled ($p = 0.035$) but did not reach significance at separate visits (data not shown). The change in lung function (FEV₁) did not correlate with any of the inflammatory parameters. FEV₁ was slightly lower in the healthy controls than in the children with asthma probably because they were not as practiced in performing forced expirations (Table 2).

Metacholine challenge

The median values for PD15 at baseline for the continuous treatment group were 137 μg , for the withdrawal group 273 μg and for the healthy controls 2800 μg .

Both the continuous treatment and the withdrawal group showed a trend toward increased PD15 values during the study, PD15 being 628 and 1428 μg , and 272 and 825 μg after 9 and 15 wk in the respective groups. There was no significant difference between the asthmatic groups at any time during the study. The three children in the withdrawal group who dropped

out because of exacerbation of asthma, performed only baseline tests.

PBE and total cell count

An increase in the number of PBE was seen in the withdrawal group, and the difference from the PBE count in the continuous group was significant at the visits (Fig. 3), after 1 ($p = 0.08$), 5, 9, 13 ($p < 0.05$) and 15 wk ($p = 0.06$). Children within the withdrawal group had a significantly increased PBE count already 1 wk after cessation of budesonide, compared with baseline, and this change stayed significant until the end of the 4-month study period ($p < 0.05$). When all the data were pooled, the change in PBE count, measured by linear regression analysis, was significantly more pronounced in the withdrawal than in the continuous treatment group ($p < 0.01$). The total number of cells remained unchanged in both groups. On a group level, asthmatic children had significantly higher PBE count than healthy children at all visits ($p < 0.05$). The number of PBE and percentage of eosinophils (PBE/total cell count) at baseline are presented in Table 2.

ECP levels in serum, sputum and NAL

When all ECP levels in S-ECP data were pooled, the overall change in the withdrawal group was significantly different from that in the continuous treatment group ($p < 0.01$). Levels of S-ECP showed a strong correlation with the number of PBE at all visits ($p < 0.05$ R : 0.4–0.8) and followed the same general trends as PBE

Table 2. Inflammatory markers at baseline, presented as medians (range)

	Continuous treatment group (n = 8)	Withdrawal group (n = 10)	Healthy controls (n = 16)
ENO (p.p.b.)	15.6 (1.8–57)	24.2 (2.6–49)	10 (6–20)
PBE ($\mu\text{l/l}$)	622 (294–2012)	416 (180–1778)	246 (50–345)
S-ECP ($\mu\text{g/l}$)	24 (15–89)	26.5 (13–144)	11.5 (3–24)
N-ECP ($\mu\text{g/l}$)	33 (10–71)	18 (13–45)	15 (2–36)
sp-ECP	167 (0–1000)	62 (0–2232)	—
FEV ₁ pred.	102 (96–132)	107 (92–116)	86 (72–108)
PD15	137 (28–175)	273 (32–2800)	2800 (700–2800)

ENO, exhaled nitric oxide; PBE, peripheral blood eosinophils; S-ECP, serum eosinophil cationic protein; N-ECP, nasal lavage eosinophil cationic protein; sp-ECP, sputum eosinophil cationic protein; FEV₁pred., forced expiratory volume in 1 s when compared with expected for age and height; PD15, provocative dose of metacholine causing a 15% fall in FEV₁.

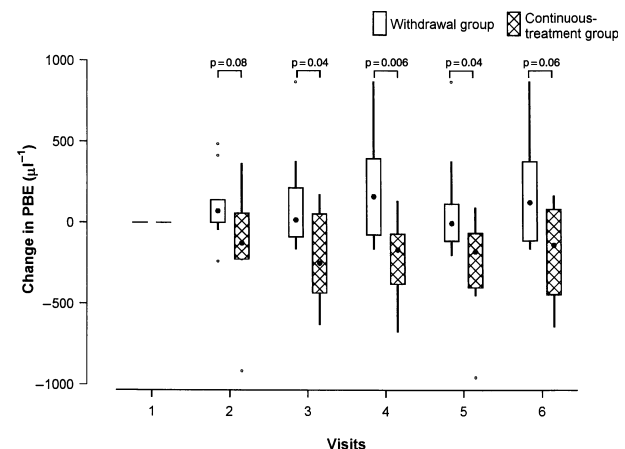


Fig. 3. Change in PBE number compared with baseline (V_1). The change from baseline values and differences between the groups are presented as median, interquartile range, minimum/maximum and outliers (dots).

although the magnitude of the changes in S-ECP was smaller.

The levels of sp-ECP were higher at baseline in children later randomized for continuous treatment than in the group randomized for withdrawal (167 and 51 $\mu\text{g/l}$, $p < 0.05$). There was no significant change within the continuous treatment or withdrawal groups at visit 5 after 13 wk (median 185 and 66 $\mu\text{g/l}$). The three children in the withdrawal group who dropped out because of exacerbation of asthma performed sputum induction at baseline only, which limited the number of children investigated twice in the withdrawal group. The skewed distribution of ECP in sputum at baseline made comparisons between the different groups impossible.

NAL was obtained from every child at every visit, but no significant trend in either group could be observed. The levels of N-ECP showed no association with the severity of disease. Nor could N-ECP distinguish between children with asthma and healthy controls.

Discussion

There is a need for controlled trials in children with asthma where different markers of inflammation are performed simultaneously and can be evaluated in relation to each other and to other indices of asthma, such as bronchial responsiveness and FEV₁.

This is to our knowledge the first double-blind, randomized, placebo-controlled study that addresses the impact of withdrawal of inhaled corticosteroids on airway inflammation in children with allergic asthma. The children were all allergic to animal dander; thus they were naturally exposed to allergens in their daily surroundings. This exposure implies a risk for allergic airway inflammation especially in those not being treated with inhaled corticosteroids (27).

The results demonstrate that withdrawal of a low dose of inhaled budesonide leads to deterioration and increased airway inflammation measured by inflammatory markers in blood, serum, and exhaled air. The present data provide evidence for the usefulness of measuring ENO, S-ECP, and PBE in clinical practice, but not for measurements of sp-ECP, NAL-ECP or PD15 methacholine.

We found a significant increase in ENO levels from 5 wk after withdrawal of budesonide treatment. The difference in ENO between the groups with asthma was already significant 1 wk after corticosteroid withdrawal, which indicates that ENO may be a useful indicator in the longitudinal assessment of asthma control.

Measurements of ENO are quick and easy to perform, thus lending itself to repeated measurements over time.

A known disadvantage of using ENO in a clinical setting is that ENO levels are strongly affected by viral airway infections, by intake of nitrate-rich food (28, 29) and by changes in anti-inflammatory treatment, even in the absence of changes in asthma symptoms and lung function. The magnitude of the change of ENO varied during our study. One possible explanation for the variability might be that the disease state changed over time because of airway infections. Another possibility is the effect of the single permitted oral dose of corticosteroids. In spite of the variability, ENO stayed increased in the withdrawal group through the 4-month study time. In a newly published open steroid withdrawal study of adults with asthma an increase in ENO over a period of 6 wk correlated with an increase in the number of subjects whose asthma went out of control, and the magnitude of the increase of ENO was predictive of asthma deterioration (30).

In the present study, the trend for ENO was the same as for PBE and S-ECP, but our material was too small for calculating the risk of relapse. However, data from our previous study indicate that PBE and/or ECP and eosinophil peroxidase (EPO) in serum can be used to estimate the short-term risk of deterioration and the need for corticosteroid treatment in cases of mild and moderate asthma (20, 31).

The method for induced sputum enables direct sampling from the airways for studying asthmatic inflammation but the clinical use of induced sputum is not without problems (25, 32). In our study, sp-ECP was measured on two occasions, at start and 13 wk after randomization but did not show any trend. One explanation could be the limited number of children investigated and also that three children in the withdrawal group had been excluded by the time of the second sputum sampling due to exacerbation of their asthma. Another possibility is that factors other than asthma influenced the outcome. The levels of sp-ECP at baseline were higher in the group randomized to continuous treatment than in the group randomized to withdrawal. This skewed allocation of subjects was seen in the levels of N-ECP but not for the other measured markers in exhaled air nor in blood or serum. A more adequate way to evaluate sp-ECP as a marker of airway inflammation would be to monitor a withdrawal period by taking sputum samples at several time points. The problem with a study that requires repeated sputum sampling

in children with mild to moderate asthma is that the procedure is both time-consuming and unpleasant for the children.

Recent studies evaluating sputum have reported difficulties in the induction and processing of sputum and propose that sputum induction is only possible in some children. In addition, the reported success rates of sputum induction in children range between 68% and 100% in different studies (12–15, 25, 32). Our experience is that sputum induction in children with mild to moderate asthma is not suitable for clinical practice, partly because of problems with low reproducibility in the sampling, but mainly because it is unpleasant for the children.

NAL procedures, unlike sputum induction are well tolerated even by infants (16–18, 27, 33). In our study, N-ECP was measured at all visits in all patients. Nevertheless no differences were seen between the withdrawal and the continuous treatment group nor between the healthy non-atopic and the atopic asthmatic children. Nor could we show any correlation between N-ECP and other markers measured simultaneously. One reason for this might have been the difficulty of getting NAL of known dilution. Some studies suggest that when N-ECP is measured, NAL-albumin/protein should be used as an internal standard (26) to estimate the degree of dilution, while others do not believe that measuring NAL-albumin is advantageous (34).

Previous prospective studies of cessation of corticosteroid therapy have often assessed asthma deterioration by means of lung function, bronchial hyperreactivity and symptoms. In a pediatric study bronchial hyperreactivity, FEV₁ and PEF deteriorated in children within 1 month after cessation of treatment (35). Studies in adults have shown conflicting results as regards changes in bronchial hyperreactivity and lung function after withdrawal of steroids (20, 36). In our study we could not demonstrate any consistent deterioration in bronchial hyperreactivity in the withdrawal group compared with the continuous treatment group, because both groups showed a trend toward increased PD15, possibly reflecting a decrease in bronchial hyperreactivity over the course of the study period. This unexpected outcome might be merely an experimental artifact; it is well known that controlled repeated follow-up *per se*, has a positive effect on bronchial reactivity (31). The three children in the withdrawal group who dropped out because of exacerbation of asthma performed only baseline tests; this may also have affected the comparison between the groups.

In conclusion both ENO and PBE are valuable markers for estimating the degree of airway inflammation in asthmatic children and can, when used together, avoid over- and under-treatment with corticosteroids.

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