

Leukotrienes and 8-isoprostane in exhaled breath condensate of children with stable and unstable asthma

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Background: Cysteinyl-leukotrienes (cys-LTs) and 8-isoprostane are biomarkers of airway inflammation and oxidative stress.

Objective: The aim of this study was to evaluate cys-LT and 8-isoprostane levels in exhaled breath condensate (EBC) of children with different degrees of asthma severity.

Methods: EBC was collected from 14 steroid-naïve children with mild persistent asthma, 13 children with stable mild- to moderate persistent asthma treated with inhaled corticosteroids (ICS), 9 ICS-treated children with unstable asthma, and 19 healthy children.

Results: In the three groups of asthmatic children, EBC concentrations of cys-LTs and 8-isoprostane were significantly higher than in control children (steroid-naïve asthmatic children: cys-LTs median, 10.8 pg/mL, $P < .001$, 8-isoprostane, 16.2 pg/mL, $P < .001$; ICS-treated stable asthmatic children: cys-LTs, 12.7 pg/mL, $P < .001$, 8-isoprostane, 18.1 pg/mL, $P < .001$; children with unstable asthma: cys-LTs, 106.0 pg/mL, $P < .01$, 8-isoprostane, 29.7 pg/mL, $P < .01$; control children: cys-LTs, 4.3 pg/mL, 8-isoprostane, 3.5 pg/mL). Cys-LT levels were higher in children with unstable asthma than in the other two asthmatic groups ($P < .05$). FE_{NO} levels were significantly higher in steroid-naïve and in children with unstable asthma compared with ICS-treated children with stable asthma ($P < .01$).

Conclusions: Our study shows that EBC cys-LTs and 8-isoprostane concentrations are higher in asthmatic children than in healthy control children, with scattered values in patients with unstable asthma. These findings suggest that EBC eicosanoid measurement may have useful clinical implications for investigating phenotype differences among asthmatic patients. (*J Allergy Clin Immunol* 2004;113:257-63.)

Key words: Asthma, children, exhaled breath condensate, cysteinyl-leukotrienes, 8-isoprostane, exhaled nitric oxide, oxidative stress

One of the most significant advances of recent years lies in our learning that asthma is not a single disease but a spectrum of phenotypes involving different cell types and mediators. We also now know that even clinically quiescent asthma can be associated with airway inflam-

Abbreviations used

EBC:	Exhaled breath condensate
Cys-LTs:	Cysteinyl leukotrienes
FENO:	Exhaled nitric oxide
ICS:	Inhaled corticosteroids
IQR:	Interquartile range

mation and oxidative stress.¹ The development and application of safe and simple methods for assessing markers of inflammation can therefore help us to gain a better understanding of the relation between the various inflammatory processes occurring in asthma² and identify patients with subclinical inflammation. Recently, it has been demonstrated that treatment targeted at controlling airway inflammation markers may be more effective in reducing asthma exacerbations than treatment based on the conventional assessment of symptoms, peak flow, and the use of β_2 -agonists.³

In the past 5 years, there has been increasing interest in measuring exhaled breath condensate (EBC) compounds in subjects with pulmonary diseases.^{4,5} EBC is feasible in children, providing access to volatile and non-volatile respiratory compounds without the need for fiberoptic bronchoscopy with BAL. The safety of EBC has recently been demonstrated also in children with asthma exacerbation, and the collection of EBC was 100% successful, starting from 4 years of age.⁶

Leukotrienes and isoprostanes are two classes of compounds that can be detected in EBC. Leukotrienes, a family of lipid mediators derived from arachidonic acid through the 5-lipoxygenase pathway, are potent constrictors and proinflammatory mediators that have been shown to have a role in asthma pathophysiology.⁷ The cysteinyl leukotrienes (cys-LTs), LTC₄, LTD₄, and LTE₄, are generated mainly by mast cells and eosinophils and are able to contract airway smooth muscle and increase mucus secretion and vascular permeability and recruit eosinophils. Increased levels of cys-LTs have been found in bronchoalveolar lavage (BAL)⁸ and induced sputum^{9,10} of asthmatic patients. Recently, higher levels of leukotrienes have also been found in EBC of asthmatic adults and children.¹¹⁻¹³

The development of specific, reliable, and noninvasive methods for measuring oxidative stress in human beings is of fundamental importance to establish the role of free radicals in lung diseases. Isoprostanes are structurally stable isomers of the conventional enzymatically derived

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TABLE I. Subject characteristics, lung function data, EBC findings, and FE_{NO} values

	Healthy subjects	Stable asthma, steroid-naïve	Stable asthma, ICS-treated	Unstable asthma
n (male subjects)	19 (8)	14 (8)	13 (9)	9 (4)
Age (y)	10.0 ± 0.9 *	9.3 ± 0.9 *	11.3 ± 0.8 *	12.2 ± 1.2 *
FEV ₁ (% pred)	97 ± 2 *	93 ± 4 *	92 ± 4 *	82 ± 3 [#]
FEF ₂₅₋₇₅ (% pred)	102 ± 8 *	88 ± 5 *	89 ± 7 *	63 ± 6 [#]
FEV ₁ /FVC	88 ± 1	86 ± 2	84 ± 3	78 ± 2
FE _{NO} (ppb)	6.0 (2.7–9.5) [†]	40.4 (19.3–50.5) [†]	14.8 (9.7–20.5) [†]	50.0 (17.4–59.0) [†]
Cys-LT (pg/mL)	4.3 (2.0–8.3) [†]	10.8 (9.3–13.1) [†]	12.7 (9.9–15.1) [†]	106.0 (4.2–218.0) [†]
8-Isoprostane (pg/mL)	3.5 (2.6–7.9) [†]	16.2 (11.7–19.1) [†]	18.1 (14.8–20.5) [†]	

*Data expressed as mean ± SEM.

[†]Data expressed as median and IQR.

prostaglandins, which are produced in vivo primarily by a free radical-catalyzed peroxidation of polyunsaturated fatty acids (lipoperoxidation). This process is a central feature of oxidative stress, which is implicated in the pathophysiology of asthma.¹⁴ Increased levels of 8-isoprostane, the best-known isoprostane, have been found in the BAL of asthmatic patients, and a further rise was demonstrated after allergen challenge.¹⁵ Higher concentrations of 8-isoprostane have also been seen in the EBC of asthmatic adults and children, revealing an association with disease severity.^{16,17}

The aim of our study was to assess cys-LT and 8-isoprostane concentrations in EBC and exhaled nitric oxide (FE_{NO}) levels in asthmatic children. In addition, we compared cys-LT, 8-isoprostane, and FE_{NO} levels in steroid-naïve asthmatic children, children with stable asthma treated with inhaled corticosteroids (ICS), and children with unstable asthma.

METHODS

Study subjects

The study included 14 steroid-naïve children with mild persistent asthma, 13 ICS-treated children with stable mild-to-moderate persistent asthma, and 9 children with unstable asthma (Table 1). Cases were recruited among patients attending the Pulmonology/Allergy Outpatient Clinic at the Pediatrics Department in Padova. The diagnosis of asthma was based on clinical history and examination, pulmonary function parameters, and response to β_2 -agonist agents, according to international guidelines.¹⁸

The steroid-naïve asthmatic children with mild persistent disease had not been treated with ICS for at least 1 month. The children with stable mild-to-moderate persistent asthma had been receiving maintenance therapy with low to medium doses of ICS (budesonide or fluticasone)¹⁸ at a constant dose for at least 2 months. Stable asthma was defined as a minimal need for short-acting β_2 -agonists, no exacerbation, and no use of systemic steroids in the last 3 months. None of the patients were receiving cys-LT receptor antagonist therapy at the time of the study. The children with unstable asthma had required one or more urgent-care visits for asthma and more than 3 oral steroid bursts in the last year. They had been taking high-dose ICS and long-acting β_2 -agonists for at least 3 months and had been regularly followed up at our center for at least 2 years. Children who had had acute upper or lower airway infection in the last 3 weeks or who had a history of poor compliance were excluded from the study. All the children were atopic, sensitized to common allergens (*Der-*

matophagoides pteronissinus and *farinae*, mixed grass pollen, *Artemisia vulgaris*, *Alternaria*, dog, and cat), as evaluated by skin prick test and/or RAST. There were no differences between the three groups of asthmatic patients in passive smoke exposure and, of the children with skin prick test positive for dog or cat dander, only one child in the stable steroid-naïve group and one in the stable ICS-treated group had a cat at home. None of the children with grass pollen allergy was studied during the grass pollen season.

The study also included 19 healthy control children who underwent EBC collection and FE_{NO} measurement. They were children with no history of asthma, atopy, or respiratory infections in the previous 4 weeks. None of the control subjects was taking any medication or smoked. Children were examined by a physician, then underwent FE_{NO} measurement, spirometry, and EBC collection.

The ethics committee of our hospital reviewed and approved the protocol, and all parents gave their informed consent.

Exhaled breath condensate collection

EBC samples were collected in a condensing device composed of two glass chambers (Incofar Srl, Modena, Italy), as previously described.^{16,17} The inner glass chamber was cooled with ice and suspended in a larger glass chamber. The children, with no nose clip, were instructed to breathe tidally through their mouths through a 2-way nonbreathing valve for 15 minutes. To minimize salivary contamination, the 2-way valve served as a saliva trap, with a 12-cm banded tube vertically positioned between the mouthpiece and the condensing device while the child's mouth remained in a position lower than the device's inlet. Children were also asked to swallow their saliva periodically. Inhalation and exhalation through the mouth was checked by the movement of the inspiratory and expiratory valves. The collected EBC samples were then immediately stored in disposable polypropylene sterile tubes (Eppendorf) at –70°C. 8-Isoprostane and cys-LTs concentrations were measured by specific enzyme immunoassay (EIA) kits (8-isoprostane, Cayman Chemical Milan, Italy; LTC₄/D₄/E₄ Amersham Pharmacia Biotech, Milan, Italy), as described elsewhere.^{11,16,17} The method was modified to lower the detection limits (8-isoprostane, 1 pg/mL; cys-LTs, 4 pg/mL); for samples below the lowest detection limits, the data were expressed as half the detection limit.¹⁷

The repeatability of EBC cys-LT and 8-isoprostane measurements on 2 different days had previously been evaluated in our laboratory¹⁷ in 16 subjects for 8-isoprostane and in 6 subjects for cys-LTs, using the method of Bland and Altman. The coefficient of repeatability was 3.2 pg/mL for both compounds, with 15 of 16 values lying within this range for 8-isoprostane and all the values lying within this range for cys-LTs. The interassay variabilities for cys-LTs and 8-isoprostane assays were <15%, in keeping with those previously reported by other authors.^{19,20}

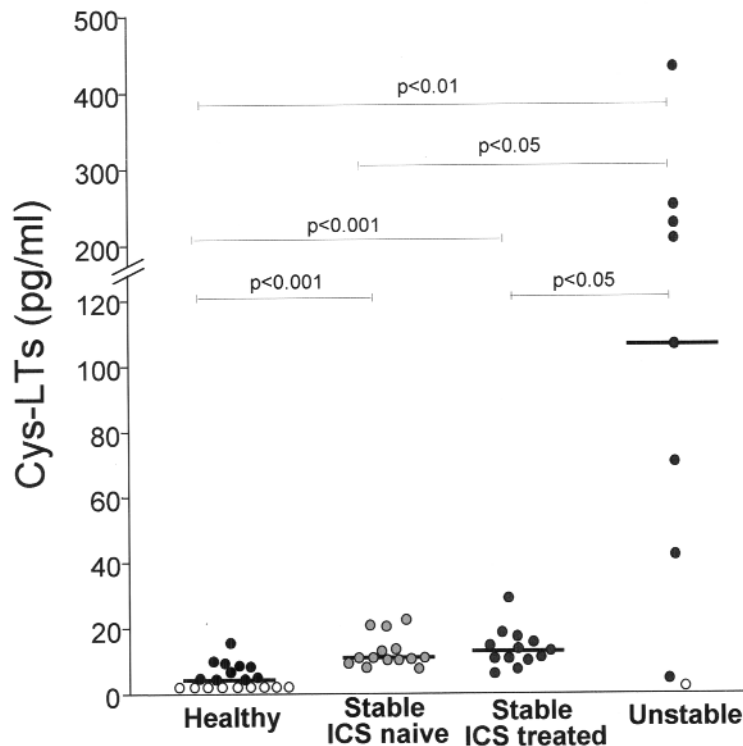


FIG 1. Cys-LTs concentrations in EBC of healthy children, steroid-naïve patients, and ICS-treated children with stable and unstable asthma. Individual data and medians are shown. Individual values below lowest detection limit are represented as open circles. *P* values were calculated by nonparametric statistical tests.

Asthma, rhinitis, other
respiratory diseases

Fractional exhaled nitric oxide measurement

FE_{NO} was measured with the NIOX system (Aerocrine, Stockholm, Sweden), using a single-breath on-line method, according to ERS/ATS guidelines for FE_{NO} measurement in children.²¹ Children inhaled NO-free air and exhaled through a dynamic flow restrictor with a target flow of 50 mL/s for at least 6 to 7 seconds.

Pulmonary function test

Pulmonary function parameters (FVC, FEV₁, FEF₂₅₋₇₅) were measured by means of a 10-L bell spirometer (Biomedin, Padova, Italy).

Statistical analysis

Data are expressed as mean ± SEM for normally distributed data and as median and interquartile range (IQR) for nonnormally distributed data. For the latter (FE_{NO}, cys-LT, and 8-isoprostane), differences among groups were analyzed by means of the nonparametric Kruskal-Wallis test, followed, where significant, by the Mann-Whitney *U* test for comparisons among groups. For normally distributed data, the differences among groups were evaluated by ANOVA, with an appropriate post hoc test (Duncan) for multiple comparisons. Correlations were evaluated by Spearman rank test. Results were considered significant at a value of *P* < .05.

RESULTS

Cys-LTs

In the three groups of asthmatic children, EBC concentrations of cys-LTs were significantly higher than those detected in healthy control children (steroid-naïve asthmatic children: median, 10.8 pg/mL [IQR, 9.3 to

13.1], *P* < .001; ICS-treated children with stable asthma: 12.7 pg/mL [9.9 to 15.1], *P* < .001; children with unstable asthma: 106.0 pg/mL [4.2 to 218.0], *P* < .01; healthy control children: 4.3 pg/mL [2.0 to 8.3]; Fig 1 and Table I). Cys-LTs levels were increased in children with unstable asthma compared with the other two asthmatic groups (*P* < .05). There was no significant difference (*P* = .66) in Cys-LTs concentrations between stable ICS-treated and steroid-naïve asthmatic children.

8-Isoprostane

EBC concentrations of 8-isoprostane were increased in the three groups of asthmatic children compared with healthy subjects (steroid-naïve asthmatic children: median, 16.2 pg/mL [IQR, 11.7 to 19.1], *P* < .001; ICS-treated children with stable asthma: 18.1 pg/mL [14.8 to 20.5], *P* < .001; children with unstable asthma: 29.7 pg/mL [4.8 to 35.1], *P* < .01; control children: 3.5 pg/mL [2.6 to 7.9]; Fig 2 and Table I). No significant differences in 8-isoprostane EBC concentrations were found among the three groups of asthmatic children.

FE_{NO}

In the three groups of asthmatic children, FE_{NO} levels were higher than in healthy control children (steroid-naïve asthmatic children: median, 40.4 ppb [IQR, 19.3 to 50.5], *P* < .001; ICS-treated children with stable asthma: 14.8 ppb [9.7 to 20.5], *P* < .001; children with unstable

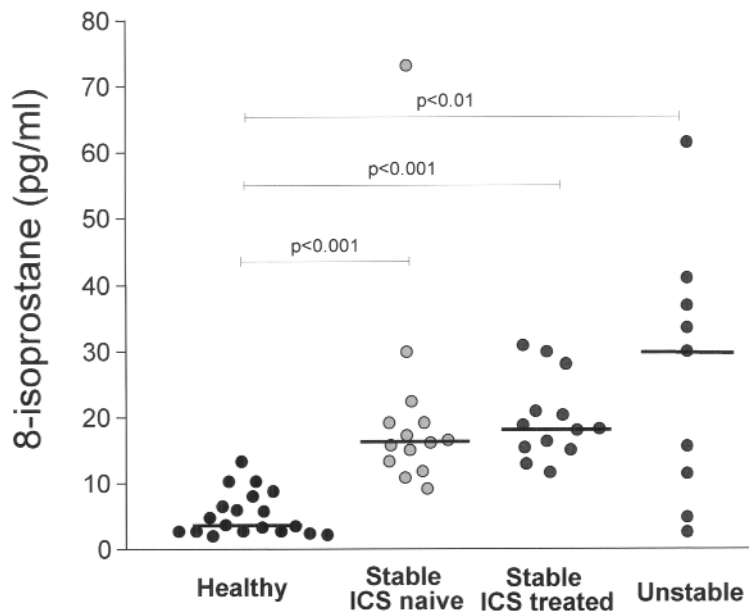


FIG 2. 8-Isoprostane concentrations in EBC of healthy children, steroid-naïve patients, and ICS-treated children with stable and unstable asthma. Individual data and medians are shown.

asthma: 50.0 ppb [17.4 to 59.0], $P < .001$; control children: 6 ppb [2.7 to 9.5]; Fig 3 and Table I). In ICS-treated children with stable asthma, FE_{NO} levels were lower than those of steroid-naïve asthmatic children ($P < .01$) and those of children with unstable asthma ($P = .01$).

Spirometric parameters

There was a significant difference in FEV_1 among the four groups studied (control children: mean $\pm$ SEM, $97\% \pm 2\%$ pred; steroid-naïve asthmatic children: $93\% \pm 4\%$; ICS-treated children with stable asthma: $92\% \pm 4\%$; children with unstable asthma: $83\% \pm 3\%$; analysis of variance, $<.05$). In children with unstable asthma, FEV_1 values were lower than those of healthy control children ($P < .05$) and those of ICS-treated children with stable asthma ($P < .05$) (Table I).

Correlations

In the whole group of asthmatic children, there was a significant correlation between EBC cys-LT and 8-isoprostane concentrations ($r = 0.47$, $P < .01$). A significant correlation between Cys-LTs and 8-isoprostane levels was found in the group of ICS-treated children with stable asthma ($r = 0.75$, $P < .01$), whereas no significant correlation was found in the other two asthmatic groups. In the group of steroid-naïve asthmatic children, there was a significant correlation between Cys-LTs and FE_{NO} ($r = 0.56$, $P < 0.05$).

DISCUSSION

Our study shows that cys-LT and 8-isoprostane concentrations in EBC are significantly higher in asthmatic children than in healthy control children. Cys-LT levels

were higher in children with unstable asthma than in steroid-naïve and ICS-treated children with stable asthma. FE_{NO} levels were also higher in asthmatic children, and more so in steroid-naïve and unstable patients than in ICS-treated stable children.

Cys-LTs appear to have a central role in asthma pathophysiology. They mediate several steps in airway inflammation, including inflammatory cell recruitment, vascular leakage, and possibly also airway remodeling. Our findings of higher EBC cys-LT concentrations in asthmatic children are in keeping with other studies that assessed cys-LT levels in EBC¹¹⁻¹³ and sputum of patients with asthma.¹⁰ In adults, EBC cys-LT levels have been found higher in patients with moderate and severe asthma than in subjects with mild asthma and control subjects,¹¹ and increased levels of EBC LTE_4 also have recently been reported in steroid-naïve patients with mild asthma.¹² In children, Csoma et al¹³ showed that EBC Cys-LT levels were higher in ICS-treated children with mild and moderate-to-severe asthma than in control children. A methodologic limit of our study is that we did not attempt to fractionate cys-LTs by HPLC to further evaluate the contribution of each class of these eicosanoids. In addition, considering that several subjects have Cys-LT values close to the limit of detection of the EIA kit that we used, we believe that more specific mass spectrometry based analytical techniques should be applied to develop and standardize reference methods for EBC measurement of these lipid mediators.

8-isoprostane is the best-characterized compound belonging to the F2 isoprostanes and can provide a reliable index of lipid peroxidation and oxidative stress in a variety of clinical settings. Isoprostanes exert important biological effects on airway and pulmonary vascular smooth mus-

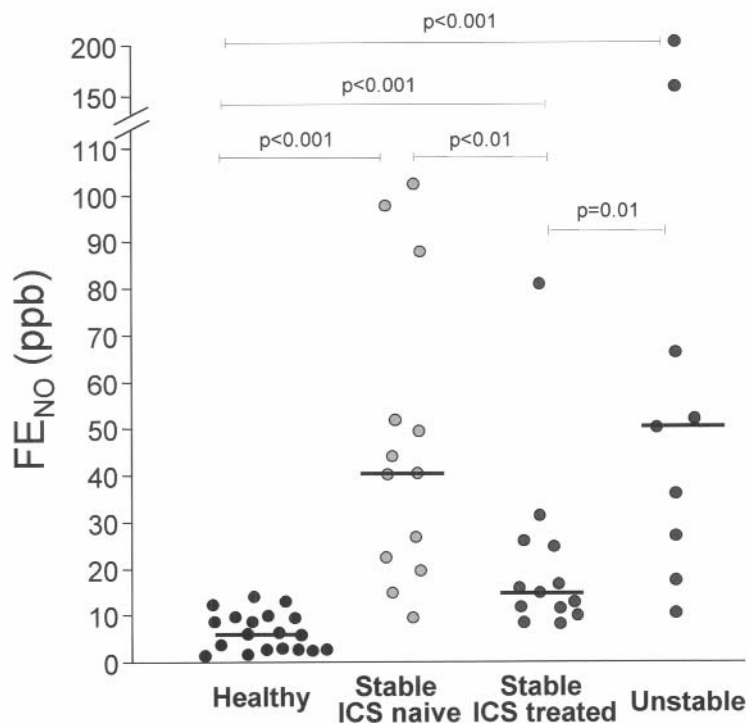


FIG 3. FE_{NO} levels in healthy children, steroid-naïve patients, and ICS-treated children with stable and unstable asthma. Individual data and medians are shown.

Asthma, rhinitis, other
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cles, epithelium, endothelium, and sensory nerves. Their action can account, in part, for the nonspecific smooth muscle hyperresponsiveness, bronchoconstriction, and edema characteristic of many lung diseases.²²

In asthmatic patients, increased levels of both systemic²³ and airway 8-isoprostane¹⁵ have been found. Montuschi et al¹⁶ recently demonstrated that 8-isoprostane concentration in EBC was higher in adult asthmatic patients than in healthy control subjects and higher in severe asthma than in milder forms. In keeping with this study, we found that asthmatic children had a marked increase in 8-isoprostane levels in EBC compared with healthy control children. This confirms evidence that airway inflammation is associated with a greater production of reactive oxygen species in the lung and supports the central role of oxidative stress in asthmatic inflammation. Of interest is that among the patients with unstable asthma, there are 2 distinct subgroups of subjects (Fig 2), one with very high 8-isoprostane levels and one with low levels, highlighting the heterogeneity of asthma phenotypes in subjects with difficult-to-control asthma.

Some clinical studies have shown a substantially heterogeneous individual patient response to antiasthma medications,²⁴ whereas conflicting data have been reported on the effect of corticosteroids on cys-LTs in vitro and in vivo. In vitro, corticosteroids have been shown to reduce cys-LT production, probably by blocking phospholipase A2.²⁵ We found no significant difference in cys-LT concentrations between ICS-naïve and ICS-treated children with stable asthma, whereas chil-

dren with unstable asthma had significantly higher cys-LT levels, despite treatment with high ICS doses. This is consistent with previous data concerning children and adults with asthma, suggesting that the cys-LT pathway of airway inflammation may be only partially controlled by ICS in vivo.^{10,11,13}

We likewise showed that 8-isoprostane levels are increased in stable ICS-treated and steroid-naïve asthmatic children, with no significant difference between the two groups, suggesting the persistence of residual inflammation and oxidative stress despite corticosteroid therapy.²⁶ It is noteworthy that median 8-isoprostane levels were 5 to 6 times higher in these two groups of children with stable asthma than in control children (Fig 2). This seems to confirm the partial effect of inhaled corticosteroids on 8-isoprostane levels, previously emphasized by other authors.^{16,23,26}

Another possible reason for the lack of difference in cys-LT and 8-isoprostane concentrations between ICS-treated and untreated asthmatic patients is that ICS may have poor access to distal airways, which are significant sites of inflammation.²⁷ Thus, inflammation may persist in the peripheral airways despite ICS therapy, becoming responsible for the increased eicosanoid production. This hypothesis is indirectly supported by recent data showing that a course of oral prednisone reduces EBC Cys-LT concentrations in asthmatic children to levels similar to those detected in control children.¹⁷ Poor compliance to ICS treatment could also explain the lack of differences in 8-isoprostane and cys-LTs between the two stable

groups of asthmatic patients. However, since FE_{NO} is extremely sensitive to ICS,^{4,26} the lower FE_{NO} values found in the ICS-treated asthmatic children compared with the steroid-naïve group suggest a good compliance with corticosteroid treatment in the treated group.

Our finding of lower FE_{NO} values in stable ICS-treated asthmatic children than in steroid-naïve asthmatic children is in keeping with previous studies showing that treating patients with mild-to-moderate asthma with ICS leads to a drop in FE_{NO} , whereas reducing the steroid dose prompts a rise in FE_{NO} .^{4,26} Consistently with previous studies on difficult asthma,^{28,29} subjects with unstable asthma had high median FE_{NO} levels despite treatment with high-dose ICS. It is worth noting that cys-LT, 8-isoprostane, and FE_{NO} values were scattered in children with unstable asthma, ranging from low values overlapping with those of control children (Figs 1, 2, and 3) to very high values, showing no significant correlation with each other, which would suggest that their measurement can detect different patterns of unstable asthma.

It has been recognized that eosinophilic airway inflammation occurs in only a proportion of patients with difficult asthma, and, for some patients, corticosteroids may have little effect on FE_{NO} and eosinophils, as well as on symptoms.²⁹ Other inflammatory cells, moreover—for example, neutrophils—may have a major role in severe asthma, or there may be a noninflammatory basis for persistent symptoms.²⁹ The normal levels of FE_{NO} or eicosanoids found in some patients with unstable asthma suggest that these children had little airway inflammation to account for their symptoms, supporting the concept of the heterogeneity of asthma. These findings are of practical clinical importance. The demonstration of persistent steroid-insensitive airway inflammation with increased cys-LT or 8-isoprostane levels may justify trials with other anti-inflammatory or antioxidative drugs.

In our study, a significant relation between cys-LTs and 8-isoprostane values was found in the group of ICS-treated children with stable asthma, whereas in the steroid-naïve asthmatic group, there was a significant relation between cys-LTs and FE_{NO} . Cys-LTs, 8-isoprostane, and FE_{NO} are thought to reflect different aspects of airway inflammation, and their levels could increase independently of each other and be affected by different factors. In addition, a different sensitivity of these markers to ICS could play a role in the different relations found in the treated and untreated groups. However, the interpretation of the relations between cys-LTs, 8-isoprostane, and FE_{NO} is hampered by the small size of the patient groups.

Exhaled breath condensate has proved to be a simple and safe technique for use in children,⁶ and, together with FE_{NO} measurement, has great potential as a noninvasive means of monitoring airway inflammation and oxidative stress in asthmatic patients. It can also be repeated several times, enabling exhaled markers of airway inflammation to be longitudinally related to other parameters of asthma control.³⁰ However, a standardization of the method³⁰⁻³² and validation of the analytical methods are

needed before EBC can be considered for clinical application.³³ An ATS/ERS task force is working in this field.

In conclusion, our study shows that EBC Cys-LT and 8-isoprostane concentrations are higher in asthmatic children than in healthy control children. Moreover, we did not find any significant difference in these eicosanoid concentrations between stable ICS-treated and steroid-naïve asthmatic children, whereas higher values were found for cys-LTs in children with unstable asthma, despite treatment with high-dose ICS. In the latter group of asthmatic children, FE_{NO} , cys-LTs, and 8-isoprostane values were scattered over a wide range and did not correlate with each other, suggesting different inflammatory patterns in children with unstable asthma. These results suggest that EBC eicosanoid measurement may have useful clinical implications in characterizing phenotype differences among asthmatic patients and for investigating the potential for antioxidant and other therapeutic strategies in asthma.

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