ORIGINAL ARTICLE

Leukotriene B_4 and 8-Isoprostane in Exhaled Breath Condensate of Children With Episodic and Persistent Asthma

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Abstract

Background: Leukotrienes and isoprostanes are biomarkers of airway inflammation and oxidative stress that can be detected in exhaled breath condensate (EBC). The aim of this study was to evaluate leukotriene B_4 (LTB_4) and 8-isoprostane levels in EBC of healthy and asthmatic children with episodic and moderate persistent asthma.
Methods: EBC was collected from 62 children aged 6 to 14 years: 22 healthy children, 30 patients with episodic asthma, and 10 patients with moderate persistent asthma, without preventive treatment at the time of enrolment.
Results: LTB_4 concentrations were higher in children with asthma than in healthy controls (50.7 pg/mL vs 13.68 pg/mL, *P*<.011). The same was true for children with moderate persistent asthma compared to children with episodic asthma (146.9 pg/mL vs 18.85 pg/mL, *P*<.0001), children with moderate persistent asthma compared to healthy controls (146.9 pg/mL vs 13.68 pg/mL, *P*<.0001), and children with episodic asthma compared to healthy controls (*P*, nonsignificant). EBC concentrations of 8-isoprostane were higher in asthmatic than in healthy children (18.3 pg/mL vs 6.59 pg/mL, *P*<.026). They were also increased in children with moderate persistent asthma compared to those with episodic asthma (36.25 pg/mL and 12.28 pg/mL, *P*<.012), and in children with moderate persistent asthma and episodic asthma compared to healthy controls (36.25 pg/mL vs 6.59 pg/mL [*P*<.0001] and 12.28 pg/mL versus 6.59 pg/mL [*P*<.0001], respectively).
Conclusion: LTB_4 and 8-isoprostane concentrations were increased in asthmatic children compared to healthy individuals, with differences detected for 2 degrees of asthma severity. Our findings suggest that EBC is a noninvasive method for airway inflammation and oxidative stress assessment.

Key words: Asthma. Children. Exhaled breath condensate. Leukotriene. Isoprostane.

Resumen

Introducción: Los leucotrienos e isoprostanos, son biomarcadores de la inflamación de la vía aérea y el estrés oxidativo, que se pueden detectar en el condensado exhalado con la respiración (CER). El objetivo, fue determinar los niveles de leucotrieno B_4 (LTB_4) y 8- isoprostano en el CER de niños sanos y asmáticos (asma episódica y persistente moderada).
Métodos: Participaron 62 niños de 6 a 14 años: 22 niños sanos, 30 con asma episódica y 10 con asma persistente moderada, sin tratamiento preventivo en aquel momento.
Resultados: Las concentraciones de LTB_4 fueron mayores en niños con asma que en los sanos (50,7 pg/ml vs 13,68 pg/ml; *P*<0,011), en niños con asma persistente moderada comparadas con asma episódica (146,9 pg/ml vs 18,85 pg/ml; *P*<0,0001), en asma persistente moderada comparadas con niños sanos (146,9 pg/ml vs 13,68 pg/ml; *P*<0,0001), y en asma episódica comparado con sanos (*P*=ns). Las concentraciones en el CER de 8-isoprostano fueron mayores en asmáticos que en sanos (18,3 pg/ml vs 6,59 pg/ml; *P*<0,026), en asma persistente moderada comparado con asma episódica (36,25 pg/ml y 12,28 pg/ml respectivamente; p<0,012), en asma persistente

moderada comparado con sanos (36,25 pg/ml y 6,59 pg/ml; *P*<0,0001) y asma episódica comparado con sanos (12,28 pg/ml vs 6,59 pg/ml; *P*<0,0001).
Conclusiones: Las concentraciones de LTB_4 y de 8-isoprostano se elevaron en asmáticos comparadas con sanos, y estos niveles fueron distintos entre los diferentes grupos de asmáticos. Esto sugiere que el CER es un método no invasivo para medir la inflamación y el estrés oxidativo en niños asmáticos.

Palabras clave: Asma. Niños. Condensado exhalado con la respiración. Leucotrieno. Isoprostano.

Introduction

Asthma is characterized by chronic airway inflammation and oxidative stress, with elevated airway inflammation often preceding the onset of symptoms or airway limitation [1,2,3].

Inflammation in asthmatic airways comprises mucosal edema, epithelial shedding, fibrosis beneath the basal membrane, eosinophil and T-cell infiltration, and an increased presence of mast cells. However, there is increasing evidence that neutrophils may play a role in more severe asthma [2].

Quantification of airway inflammation is difficult since it requires invasive techniques or the measurement of biomarkers in plasma or urine that reflect systemic rather than lung inflammation. Bronchoscopy with biopsy remains the gold standard for assessing airway inflammation, but its invasiveness makes it unethical as a routine method, particularly in children. Sputum induction is less invasive, though it is particularly difficult to apply in young children. Furthermore, the technique actually induces neutrophilic airway inflammation. Exhaled breath condensate (EBC) collection and fraction of exhaled nitric oxide (FE_{NO}) measurement are completely noninvasive methods for the subsequent analysis of airway secretions [4]. A number of inflammatory markers found in EBC have been investigated as possible biomarkers of disease activity.
EBC utilization is feasible in children, providing access to volatile and nonvolatile respiratory compounds without the need for bronchoscopy [1,5]. The safety of EBC has also been demonstrated in children with asthma exacerbation [6].

Exhaled air basically consists of water vapor and substances that reflect the functional status of the lung and other tissues. These substances (both volatile and nonvolatile) can be analyzed by condensing exhaled air through a cooling unit. The collection of EBC is a simple, noninvasive technique that requires minimal cooperation on the part of the patient (of particular interest in pediatric patients). It is reproducible over time, involves short collection periods, does not require special facilities or specially trained personnel, and can be performed with portable equipment and at a low cost. The procedure was first developed in the 1980s in the former Soviet Union, but became widely known in the late 1990s [7,8].

Leukotrienes and isoprostanes are 2 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 play a role in asthma physiopathology [5].

Neutrophils release LTB_4 in response to various activating stimuli, and also have a high density of cell-surface LTB_4 receptors. LTB_4 exerts potent chemotactic action upon neutrophils, with no significant effect on airway muscle [3].

F2-isoprostanes are considered to be markers of oxidative stress. They are synthesized from arachidonic acid by free radical catalyzed lipid peroxidation; their synthesis is fundamentally independent of cyclooxygenase (COX) action. The most prevalent isoprostane in humans is 8-epi-PGF2-α, also known as 8-isoprostane.

Increased levels of leukotrienes, and 8-isoprostane in association with disease severity, have been found in EBC of asthmatic adults and children [1,5]. Levels of 8-isoprostane are approximately twice as high in patients with mild asthma compared to healthy individuals and three times higher in patients with severe asthma, irrespective of treatment with corticosteroids [9].

The aim of this study was to assess LTB_4 and 8-isoprostane concentrations in EBC in asthmatic and healthy children. In addition, we compared LTB_4 and 8-isoprostane levels in children with episodic (intermittent or frequent) and moderate persistent asthma.

Materials and Methods

Participants

Three groups of children aged 6 to 14 years were studied. These included healthy nonatopic children (n=22), children with episodic asthma (n=30), and children with moderate persistent asthma (n=10) (Table 1). The children with asthma were recruited from the allergology unit of Hospital General Universitario, in Valencia, Spain. There was no significant difference in age between the patient groups.

The diagnosis of bronchial asthma was based on the Global Initiative for Asthma guidelines [10], ie, recurrent episodes of wheezing, cough and/or shortness of breath. Asthma severity was classified according to the SENP-SEICAP consensus statement [11]. Briefly, children with episodic asthma had symptoms less often than once every 5 to 6 weeks; while they were not taking regular medication, they used inhaled ß$_2$-agonists as needed for symptom relief. Children with moderate persistent asthma had symptoms more than once every 4 to 5 weeks. They were not taking any regular medication other than ß$_2$-agonists as needed for

symptom relief until examination, when we recommended preventive treatment because of their progression from episodic to moderate persistent asthma.

All the patients were atopic and sensitized to *Dermatophagoides* and/or *Alternaria*, both common perennial airborne allergens. Atopy was assessed by skin prick tests for common allergens and specific immunoglobulin (Ig) E determination.

Healthy participants included children seen at the allergology unit due to a suspected drug reaction and children of personnel from the unit. They had no personal or family history of asthma or atopy and had been free of respiratory infections in the preceding 4 weeks.

The children were examined by a physician, and then underwent FE_{NO} measurements, spirometry, and EBC collection. Excluded from the study were children who had used oral/inhaled corticosteroids or montelukast in the previous 4 weeks, or nonsteroidal anti-inflammatory drugs in the previous 2 weeks.

The protocol was approved by the hospital ethics committee and informed consent was obtained from all children recruited and their parents.

Study Design

After collecting participant details, we performed baseline spirometry, bronchodilation testing, skin prick tests, and exhaled FE_{NO} measurements. We then collected EBC.

Pulmonary Function Tests

Pulmonary function parameters (forced vital capacity [FVC], forced expiratory volume in 1 second (FEV_1), mild forced expiratory flow (FEF_{25-75}) were measured by means of a computer-assisted spirometer (Datospir 2000; Silbel S.A., Barcelona, Spain). The best of 3 measures, expressed as a percentage of predicted values, was chosen. Bronchodilation testing was performed according to international guidelines using a metered salbutamol dose of 400 μg. Reversibility was considered if an increase of at least 12% in FEV_1 from baseline was achieved [12,13].

EBC Collection

EBC samples were collected with a specially designed condensing chamber (Anacon; Biostec, Valencia, Spain). Exhaled air entered and left the chamber through 1-way valves at the inlet and outlet, thus keeping the chamber closed. Participants breathed tidally through a mouthpiece connected to the condenser for 20 minutes while wearing nose clips. They were asked to swallow their saliva periodically. A temperature of –8ºC inside the condensing chamber throughout the collection time produced immediate sample freezing. The collected EBC samples were stored at –70ºC before eicosanoid measurements.

LTB_4 and 8-isoprostane were measured with a specific enzyme immunoassay (EIA) kit (Cayman Chemical Company; Ann Arbor, Michigan, USA). The detection limits were 4.5 pg/mL for LTB_4 and 1 pg/mL for 8-isoprostane.

FE_{NO} Measurement

FE_{NO} was measured using the Niox-Mino analyzer (Aerocrine, Solna, Sweden) and the single breath online method in accordance with the American Thoracic Society guidelines for FE_{NO} measurement in children [14].

Statistical Analysis

Median and interquartile ranges were used as summary measures because of the asymmetric distribution of LTB_4 and 8-isoprostane concentrations. Mann-Whitney testing was used to compare groups. The correlations between LTB_4, 8-isoprostane, and FE_{NO} were determined by nonparametric Spearman correlation analysis. Statistical significance was set at $P<.05$.

Table. Participant Characteristics, Lung Function Data, EBC Findings and FE_{NO} Values[a]

	Healthy Participants	Patients With Episodic Asthma	Patients With Persistent Asthma
No. (males)	22 (12)	30 (19)	10 (4)
Age, y	10 (9-11.2)	11 (9-12)	11.5 (8.75-14)
FEV_1, % predicted	110 (98-113)	96 (87-108)	88.6 (85-91)
FEF_{25-75},% predicted	100 (94.5-110.3)	84 (75-102)	79.5 (73.8-86.3)
FEV_1/FVC	102 (99.7-105.2)	89 (85-100.5)	98.5 (95-100.5)
FE_{NO}, ppb	14 (11-18.5)	35 (21.8-49.3)	49 (27-72.8)
LTB_4, pg/mL	6.6 (5.4-12.7)	11.7 (5.2-26.5)	133.8 (68.5-151.7)
8-isoprostane, pg/mL	4 (2-6)	12.5 (8.3-15.8)	29.7 (12-38.9)

Abbreviations: FE_{NO}, fraction of exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; LTB_4, leuokotriene B_4; EBC, exhaled breath collection; ppb, parts per billion.
[a]Values are expressed as medians (25th-75th percentile) unless otherwise expressed.

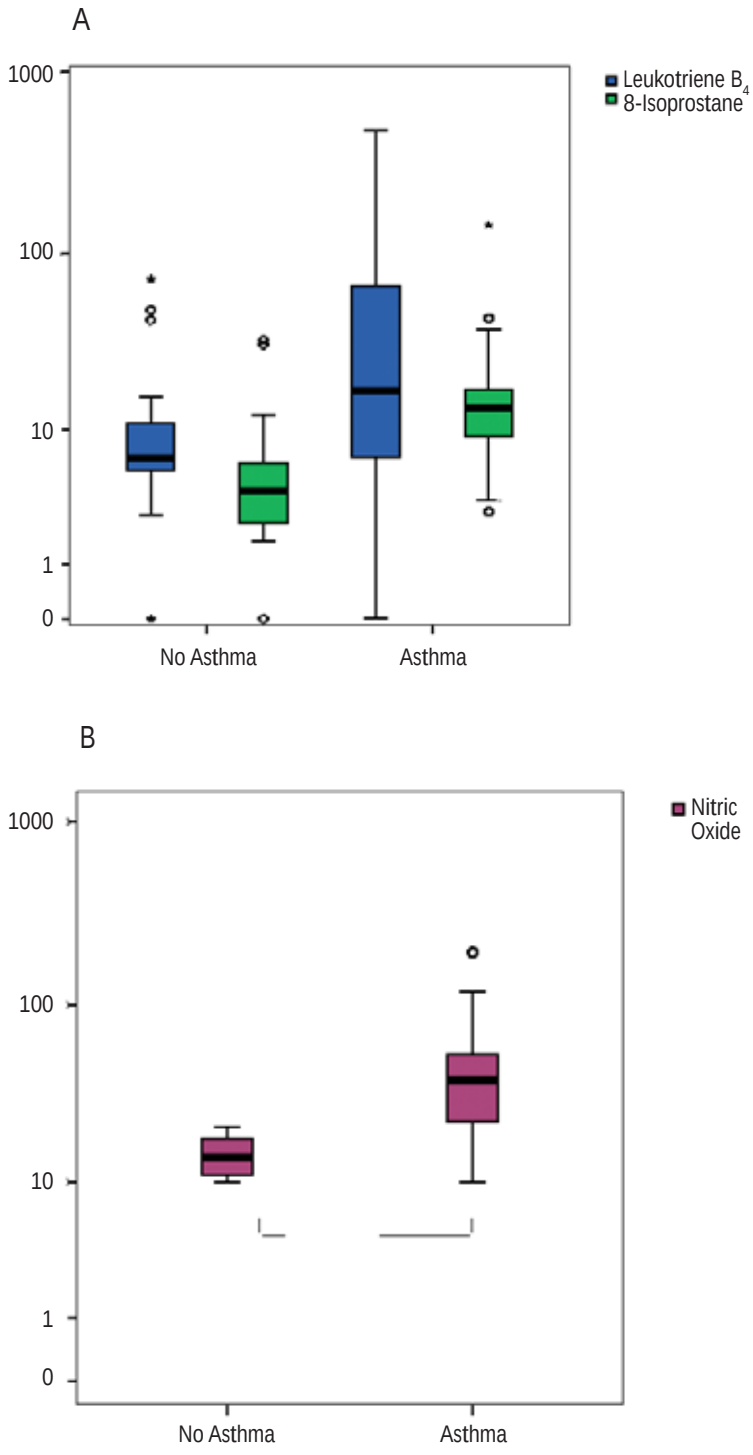

Figure 1. A, Leukotriene B_4, 8-isoprostane values (pg/mL) in asthmatic and healthy children, expressed as median (25th-75th percentiles).
B, Fraction of exhaled nitric oxide (parts per billion) in asthmatic and control children expressed as median (25th-75th percentiles) (*P*<.001).

Results

LTB_4 and 8-isoprostane concentrations were detectable in all healthy and asthmatic children.

LTB4

Mean (SD) EBC levels of LTB_4 were significantly increased in children with asthma compared to healthy chil dren (50.73 [82.86] pg/mL vs 13.68 (18) pg/mL, *P*<.011). LTB_4 was also significantly increased in children with moderate persistent asthma (146.9 [121.3] pg/mL) compared to both children with episodic asthma (18.85 [20.6] pg/mL], *P*<.0001) and healthy controls (13.68 [18] pg/mL, *P*<.0001). Children with episodic asthma also had higher LTB_4 levels than controls but the difference was not significant.

8-Isoprostane

Mean (SD) EBC concentrations of 8-isoprostane were increased in asthmatic children compared to healthy participants (18.3 [22.36] pg/mL vs 6.59 [8.92] pg/mL, *P*<.026). 8-isoprostane was also significantly increased in children with moderate persistent asthma compared to children with episodic asthma (36.25 [29.97] pg/mL vs 12.28 [5.4] pg/mL, *P*<.012), in children with moderate persistent asthma compared to healthy controls (36.25 [29.97] pg/mL vs 6.59 [8.92] pg/mL, *P*<.0001), and in children with episodic asthma compared to healthy controls (12.28 [5.4] pg/mL vs 6.59 [8.92] pg/mL, *P*<.0001).

Correlations

We observed a significant correlation between EBC concentrations of LTB_4 and 8-isoprostane for the asthmatic group as a whole (r=0.89, *P*<.0001), for the moderate persistent asthma group (r=0.9, *P*<.0001), and for the control group (r=0.51, *P*<.017). Levels were not significantly correlated in the episodic asthma group.

No significant correlation was found between FE_{NO} or FEV_1 and either LTB_4 or 8-isoprostane.

Discussion

There is growing evidence that neutrophils play an important role in the pathogenesis of asthma during exacerbations and in severe asthma. LTB_4 exerts potent chemotactic action upon airway neutrophils, and moreover acts as a mediator inhibiting neutrophil apoptosis in vitro. Other studies have shown neutrophils in asthmatic patients to be activated, with cells showing

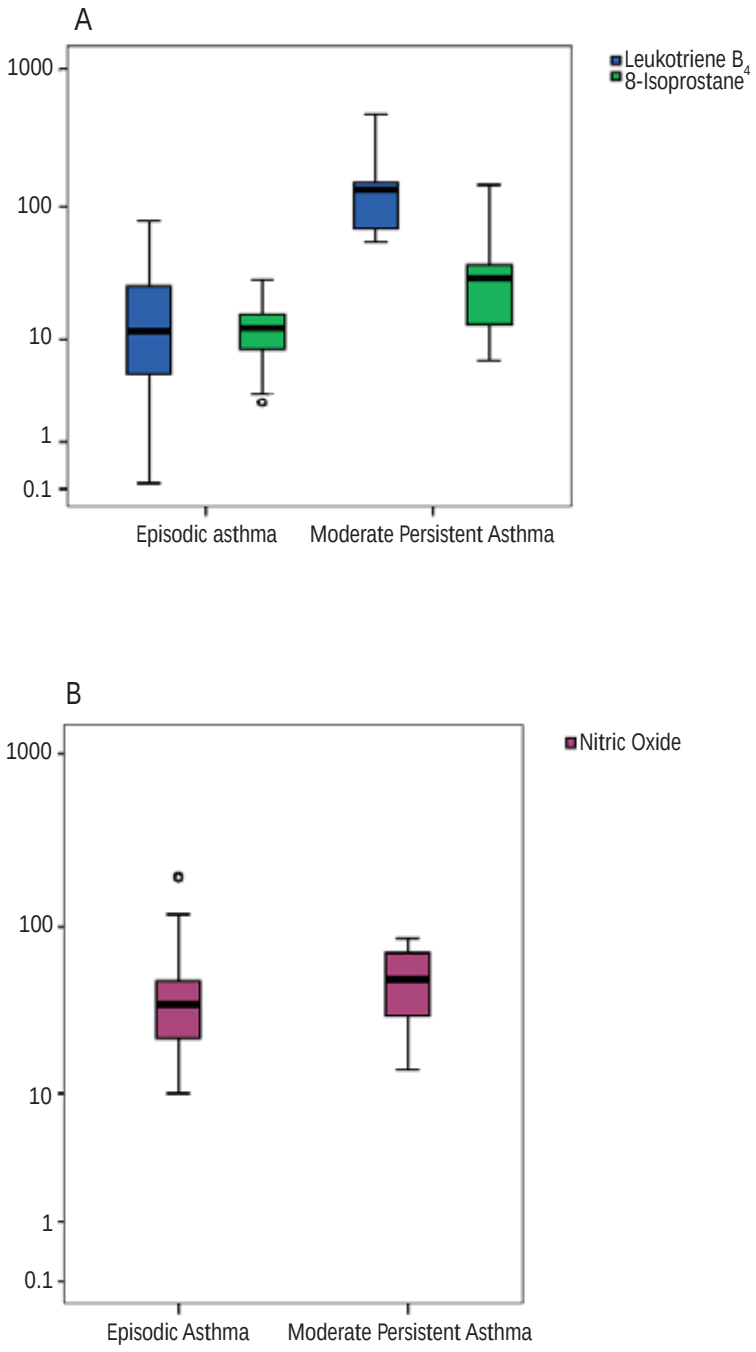

Figure 2. A, Leukotriene B_4 and 8-isprostane values (pg/mL) in patients with episodic and moderate persistent asthma, expressed as median (25th-75th percentiles).
B, Fraction of exhaled nitric oxide (parts per billion) in patients with episodic and moderate persistent asthma, expressed as median (25th-75th percentiles) (*P*, nonsignificant).

increased complement receptor expression and activity of the lipoxygenase pathway [5,15,16].

In our study, LTB_4 levels in children with episodic asthma were not elevated, pointing to a relationship between LTB_4 and more severe asthma.

In agreement with our findings, Csoma et al [16] showed cysteinyl-leukotrienes (cys-LTs) and LTB_4 to be elevated in EBC of children with mild persistent asthma and moderate asthma, but not in those with mild intermittent asthma. However, other studies have reported increased levels in bronchoalveolar lavage samples from children with mild symptomatic asthma [17,18]. These differences are logical, since in our series the patients were asymptomatic at the time of the study and had not experienced recent asthma episodes. Furthermore, because the studies did not use the same classification systems for patients with less severe asthma, the inclusion criteria may have differed in each case.

Regardless of asthma severity, it is widely agreed that LTB_4 levels are higher in asthmatic children than in healthy children. Mondino et al [4] found LTB_4 to be higher in patients both with and without corticosteroid treatment than in healthy children or nonasthmatic atopic patients. Cap et al [19], in turn, found LTB_4 to be increased 1.6-fold in asthmatics with respect to healthy subjects.

In 2005, Montuschi et al [2] concluded that LTB_4 was increased in asthmatic children without corticosteroid treatment, but not in nonasthmatic atopic children, and proposed that corticosteroid use might lower these levels. In 2004, Bodini et al [20] reported higher LTB_4 levels when patients were exposed to an allergen than after exposure was suppressed.

Although no specific cutoff has been established for differentiating between LTB_4 levels in healthy and asthmatic individuals, there is growing evidence of the usefulness of the EBC technique in measuring bronchial inflammation, and of the applicability of LTB_4 as an expression of neutrophil-mediated bronchial inflammation.

We have also analyzed 8-isoprostane, a metabolite of arachidonic acid [21] synthesized mainly via a nonenzymatic pathway catalyzed by free radicals that act upon arachidonic acid. This metabolite is an oxidative stress marker that can be used as an inflammatory marker. It is stable, specific to lipid peroxidation, synthesized in vivo, and biologically active.

The present study shows 8-isoprostane to be detectable in the EBC of children, with significantly higher values found in asthmatic individuals than in healthy children. We also found 8-isoprostane in the condensate to be significantly increased in children with moderate persistent

asthma compared to both children with episodic asthma and healthy children, as well as in children with episodic asthma compared to healthy children.

Similar results were published by Zanconato et al [5] in a study in which 8-isoprostane levels were higher in asthmatic children than in healthy individuals. Furthermore, these levels increased with asthma severity. The results of Mondino et al [4] also coincide with our findings.

Reports of responses in association with corticosteroid therapy, vary. Baraldi et al [1], for example, found 8-isoprostane levels to be higher in asthmatic than in healthy children. While these levels decreased following corticosteroid administration, they were still higher than in healthy children. In another study, however, Baraldi et al [22] reported no differences in 8-isoprostane levels between asthmatic patients with or without corticosteroid treatment, an observation coinciding with data published by Shahid et al [23].

The above studies support the evidence that airway inflammation is associated with the production of reactive oxygen species in the lungs, explaining the role of oxidative stress in asthma. As a result, corticosteroids would be only marginally effective in lowering such stress.

In our study we observed a correlation between 8-isoprostane and LTB4 in the asthmatic group, the control group, and the moderate persistent asthma group.

In 2004, Zanconato et al [5] reported a significant correlation between cys-LT and 8-isoprostane concentrations in EBC of asthmatic children and children with stable asthma treated with corticosteroids.

The above authors also observed a significant correlation between FE_{NO} and cys-LT in a group of asthmatic children. Other authors [24], in turn, have reported a positive correlation between cys-LT and both FE_{NO} levels and FEV_1. However, in our series no correlation was found between LTB_4 and respiratory function parameters. This difference is probably due to the fact that different criteria were used to define the asthmatic groups in the 2 studies. This difference in results is logical since LTB_4, 8-isoprostane, and FE_{NO} reflect different aspects of airway inflammation, and their levels could increase independently of each other, and be affected by different factors. In addition, lung inflammation is not always reflected in clinical manifestations or lung function measurements.

In conclusion, the present study shows LTB_4 and 8-isoprostane levels in EBC to be higher in asthmatic children than in healthy controls and also to be higher in moderate persistent asthma than in episodic asthma. Furthermore, 8-isoprostane levels were higher in the EBC of children with episodic asthma and moderate persistent asthma than in healthy controls. A significant correlation was observed between LTB_4 and 8-isoprostane in all groups except the episodic asthma group.

These initial results appear to confirm the usefulness of determining these inflammatory mediators in EBC for the diagnosis and evaluation of the severity of pediatric asthma, and also for investigating the relationship with oxidative stress and possible treatment control. However, further studies are needed to standardize the technique and validate the methods used to measure the mediators in EBC in order to define a cutoff for differentiating between healthy children and children with asthma, and between different degrees of asthma severity.

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