

Elevated exhaled leukotriene B₄ in the small airway compartment in children with asthma



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ABSTRACT

Background: Inflammatory processes in the asthmatic lung involve the large and small airway and alveolar sites. Leukotriene B₄ (LTB₄) is an important disease marker, but its role in inflammation of the small airways in asthma has not been established yet.

Objective: To distinguish between large and small airway or alveolar LTB₄ concentrations in children with asthma using the new technique of fractionated exhaled breath condensate sampling.

Methods: Sixty-eight children (9–17 years old, 33 children with asthma and 35 controls) underwent fractional exhaled nitric oxide (FeNO) measurements, lung function testing, and collection of fractionated exhaled breath condensate using a capnograph-based approach. The LTB₄ concentrations in the small airway or alveolar and large airway fractions were correlated to disease status, lung function impairment, and clinical parameters.

Results: Children with asthma had significantly higher LTB₄ concentrations in the small airway or alveolar fraction than controls (5.58 pg/mL; 95% interquartile range [IQR], 2.0–11.77 pg/mL; vs 2.0 pg/mL; 95% IQR, 2.0–6.2 pg/mL; *P* = .003). No difference was found between the groups in the large airway fraction. Children with obstructive lung function impairment (forced expiratory volume in 1 second *z* score < −1.65) had increased small airway or alveolar LTB₄ concentrations compared with children without impairment (2.0 pg/mL; 95% IQR, 2.0–9.21 pg/mL; vs 18.32 pg/mL; 95% IQR, 3.7–23.02 pg/mL; *P* = .04). Children with asthma but without pathologic obstructive lung function still had higher LTB₄ concentrations than controls (5.57 pg/mL; 95% IQR, 2.00–10.60 pg/mL; vs 2.00 pg/mL; 95% IQR, 2.00–6.20 pg/mL; *P* = .01).

Conclusion: LTB₄ is detectable and elevated in the small airway or alveolar fraction of exhaled breath condensate in pediatric asthma. Because of the possibility of detecting elevated levels in patients without lung function impairment in controlled disease, it may be used as a noninvasive marker of small airways disease; however, future long-term studies are needed.

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Introduction

Asthma is an inflammatory disease with involvement of the large airways and the small airway or alveoli.^{1,2} Although progression and therapy are mostly evaluated by overall parameters such as lung function testing and fractional exhaled nitric oxide

(FeNO), determining predominant sites of inflammation might be helpful for a better understanding of the disease and improved therapy monitoring.³ Recent data suggest that increased distal inflammation, measured by alveolar nitric oxide or hydrogen peroxide might be related to worse asthma control.^{3,4} So far, there is sparse information on additional biomarkers measured in the small airway and alveoli, especially noninvasively.

Leukotriene B₄ (LTB₄) is a proinflammatory lipid mediator that is important for inflammatory cell recruitment and the subsequent development of airway hyperresponsiveness. Released by neutrophils and mast cells, LTB₄ acts as a chemoattractant and inflammatory mediator.^{5,6} Elevated levels of LTB₄ have been reported in

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Table 1
Characteristics of the study participants

Characteristic	Patients with asthma (n = 33)	Controls (n = 35)
Age, median (IQR), y	14 (12–15)	15 (13–17)
Male sex ^a	19	18
Atopy	26	14
Allergic rhinitis	17	4
Atopic dermatitis	7	1
Treatment		
ICS		
Total	22	
Dosage, median (IQR), µg	200 (0–400)	
Budesonide	8	
Fluticasone	13	
Long-acting bronchodilator		
Total	9	
Salmeterol	8	
Formoterol	1	
Montelukast	5	
FEV ₁ , z score	0.06 (–0.66 to 0.49)	0.32 (–0.43 to 0.75)
FEV ₁ , % predicted	100.7 (92.2 to 105.8)	103.7 (94.95 to 108.75)
FVC, z score	0.1 (–0.99 to 0.41)	–0.04 (–0.63 to 0.67)
FVC, % predicted	101.2 (88.5 to 104.8)	99.5 (92.6 to 108.05)
FEF _{25–75} , z score	–0.32 (–1.00 to 0.25)	0.00 (–0.45 to 0.45)
sRAW _{tot} , kPa/s	0.57 (0.42 to 0.71)	0.54 (0.36 to 0.73)
RV, % predicted	100.2 (82.15 to 126.00)	108.9 (65.90 to 139.28)
ITGV, % predicted	106.5 (92.05 to 114.90)	107.0 (91.40 to 116.00)
RV/TLC, %	24.27 (20.78 to 27.00)	23.3 (19.40 to 31.09)
FeNO, ppb	16 (11 to 41)	10 (7 to 13)
ACT score		
<12 years old (n = 3)	18 (12 to 27)	
≥12 years old (n = 30)	23 (19 to 25)	
LTB ₄ large airway fraction, median (IQR), nmol/L	5.20 (2.00 to 13.71)	4.03 (2.0 to 9.03)
LTB ₄ small airway or alveolar fraction, median (IQR), nmol/L	5.58 (2.00 to 11.77)	2.00 (2.00 to 6.20)

Abbreviations: ACT, Asthma Control Test; FeNO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; ICS, inhaled corticosteroid; IQR, interquartile range; ITGV, intrathoracic gas volume; LTB₄, leukotriene B₄; FEF_{25–75}, forced expiratory flow at 25%–75%; sRAW_{tot}, specific total airway resistance; RV, residual volume; RV/TLC, residual volume/total lung capacity.

^aData are presented as number of patients unless otherwise indicated.

children with asthma and are related to asthma severity and suggested as a biomarker with high sensitivity and specificity for the diagnosis of asthma.^{7,8} Inhaled corticosteroid treatment and treatment with montelukast reduces LTB₄ concentrations in patients with asthma.^{9,10} Increased amounts of neutrophils and mast cells in the peripheral lung of patients with asthma and the inflammatory role of LTB₄ released from mast cells in experimental asthma hint to an important role for peripheral LTB₄ in asthmatic airway inflammation.^{6,11} So far, there are no data on whether LTB₄ plays a role in small airway disease and distal inflammation in asthma.

Exhaled breath condensate (EBC) sampling is an established technique to detect soluble inflammation markers in the lung.¹² With the new technique of fractionated EBC sampling, it is possible to distinguish between the large airway and the small airway or alveolar fraction of the lung by dividing each collected exhaled breath into 2 parts.^{4,13}

In this study, we examined for the first time, to our knowledge, LTB₄ in fractionated EBC in children, using a new capnograph-based method to distinguish between the gas exchange (termed *small airway* or *alveolar*) and the nongas exchange (termed *large airways*) fraction. Our primary aim was to examine the distribution of large airway and small airway or alveolar LTB₄ in fractionated EBC and compare the concentrations between patients with asthma and controls. In addition, we correlated the results to disease status, disease control, lung function impairment, and FeNO. We hypothesize that small

airway or alveolar LTB₄ concentrations are elevated in patients with asthma and are related to lung function impairment and/or asthma control.

Methods

Study Participants

Patients with asthma were recruited from the asthma outpatient clinic of the Children's University Hospital. Asthma was diagnosed clinically when children had episodic cough, breathlessness, and wheeze responsive to bronchodilators according to international and American Thoracic Society criteria.

Controls with no history of chronic cough, wheezing, or other pulmonary symptoms and without any chronic disease involving the immune system (eg, Crohn disease, diabetes mellitus, and rheumatic diseases) were recruited in various outpatient clinics. Participants' age ranged from 9 to 17 years. Detailed information about the study population is available in Table 1. Patients who were actively smoking or had an acute respiratory infection during the previous 2 weeks were excluded.

Study Design

Patients and controls completed the study protocol in the following order within 4 hours. They underwent clinical examination and then filled out an asthma questionnaire. Atopic sensitization was tested by the radioallergosorbent test or skin prick test as previously described.⁴ Subsequently, patients underwent FeNO measurement, lung function testing, and collection of fractionated EBC. This study was designed as a cross-sectional study and was approved by the local ethics committee of the Martin-Luther-Universität Halle-Wittenberg. Written consent was obtained from the participant's parents and age-appropriate consent from the children themselves.

Asthma Questionnaire

To evaluate disease control for children 12 years or older, the Asthma Control Test (ACT) was used; for children younger than 12 years, the Childhood ACT was used.^{14,15}

Lung Function Tests

Body plethysmography and spirometry (Masterlab, Jaeger, Wuerzburg, Germany) were performed as previously reported for all study participants.¹⁶ The z scores for spirometric values were calculated with the desktop calculator of the Global Lung Initiative¹⁷; a forced expiratory volume in 1 second (FEV₁) value below –1.65, which is defined as the lower limit of normal by the American Thoracic Society, European Respiratory Society, and the Global Lung Initiative, was defined as obstructive lung function impairment.

FeNO Measurements

FeNO was measured using the NIOX MINO (Aerocrine, Solna, Sweden) according to the manufacturer's recommendations (flow rate of 50 mL/s).

Fractionated EBC

Fractionated EBC was collected using ECoScreen 2 (Filt GmbH, Berlin, Germany). In this system, exhaled air is guided through the first collection chamber until a set exhaled breath volume is obtained. Afterward, the air of the second part of the exhalation is passed through a parallel second chamber. The volume threshold was determined with individual capnograph measurements for each patient and control. As described by Möller et al,¹³ the exhaled breath volume at which the carbon dioxide exhalation curve

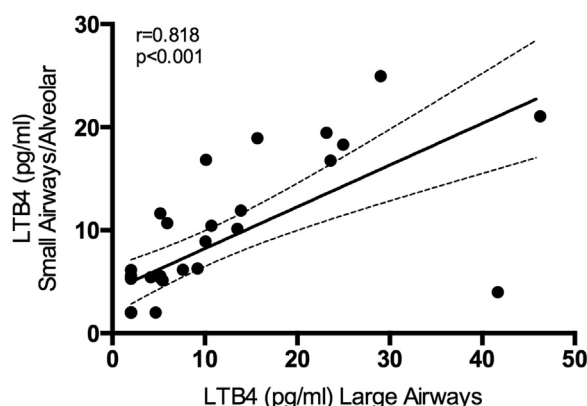


Figure 1. Leukotriene B₄ (LTB₄) concentrations of large airway and small airway or alveolar fraction of asthmatic patients are correlated ($r = 0.818$, $P < .001$).

reached its concentration plateau was considered the threshold between the large airway fraction and the small airway or alveolar fraction.

Enzyme-Linked Immunosorbent Assay

Samples were immediately stored at -80°C . LTB₄ concentrations were analyzed by a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, Michigan). The detection limit was 4 pg/mL. Values below the detection limit were expressed as half the detection limit.¹⁸

Statistical Analysis

By including 33 patients with asthma and 35 controls in our study, we achieved a minimal sufficient power of 80% (with a 2-sided test with a clinical relevant probability of 70%, $\alpha = .05$) for comparison of LTB₄ levels between the 2 groups.

Data were processed using SPSS statistical software, version 21 (SPSS Inc, Chicago, Illinois). Values are expressed as median and interquartile range (IQR). Correlations were measured using the Spearman rank correlation. Comparisons between groups were made using the Mann-Whitney test. Differences of LTB₄ concentration between the fractions were analyzed by the Wilcoxon signed-rank test, comparing each single related sample pair (paired difference test). Results with $P < .05$ were considered statistically significant. P values are exploratory for the achieving of the additional aims (subgroup analysis).

Results

Patient Characteristics

A total of 33 patients with mild to moderate persistent asthma and 35 controls participated in this study. There was no significant difference in age and sex distribution. For complete information, see Table 1.

LTB₄ Concentrations in the Large and Small Airways

Comparing each single related sample pair of LTB₄ concentrations in all participants by Wilcoxon test revealed that values of the large airways were mostly higher or equal compared with those of the small airway or alveoli. However, the difference did not reach statistical significance ($P = .05$). Analyzing subgroups of patients with asthma or controls did not change the results ($P = .38$ and $P = .06$). Large airway and small airway or alveolar concentrations were highly correlated ($r = 0.818$, $P < .001$, Fig 1).

LTB₄ Concentrations in Patients With Asthma and Controls

Patients with asthma had significantly higher LTB₄ concentrations in the small airway or alveolar fraction than controls (5.58 pg/mL; IQR, 2.0–11.77 pg/mL; vs 2.0 pg/mL; IQR, 2.0–6.2 pg/mL; $P = .003$, Fig 2A). No difference was found between patients with asthma and controls regarding the LTB₄ concentrations of the large airway fraction ($P = .18$, Fig 2B).

The subgroup of patients with asthma who were prescribed inhaled corticosteroids did not have significantly different LTB₄ values in the large airway or in the small airway or alveolar fraction (Wilcoxon test, $P = .87$ and $P = .69$, respectively) compared with patients with asthma without prescribed inhaled corticosteroids.

LTB₄ and Atopy

Comparing the LTB₄ concentrations between atopic and non-atopic patients reveals significantly higher LTB₄ values in atopic patients in both the large airway (Fig 3B) and the small airway or alveolar fractions (Fig 3A) compared with nonatopic patients (large airway fraction: 6.58 pg/mL; IQR, 2.00–13.43 pg/mL; vs 2.00 pg/mL; IQR, 2.00–7.64 pg/mL; $P = .02$; small airway or alveolar fraction: 5.59 pg/mL; IQR, 2.00–10.68 pg/mL; vs 2.00 pg/mL; IQR, 2.00–4.88 pg/mL; $P = .004$). In addition, analyzing the subgroup of asthmatic patients, differences were also present between atopic and nonatopic patients.

Correlation to Lung Function Parameters and Asthma Control

Patients with obstructive lung function impairment (FEV_1 z scores < -1.65 , $n = 5$) had significantly higher LTB₄ levels in the small airway fraction than patients without lung function impairment ($n = 63$; 18.32 pg/mL; IQR, 3.70–23.02 pg/mL; vs 2.00 pg/mL; IQR, 2.00–9.21 pg/mL; $P = .04$). Differences in the large airway fraction were also present but not significant ($P = .052$). Patients with an forced expiratory flow at 25%–75% (FEF_{25-75}) z score less than -1.65 ($n = 4$) had significantly higher LTB₄ concentrations in both large airways and small airway fraction compared with asthmatic patients with values above -1.65 ($P = .007$ and $P = .005$, respectively).

In the group of patients without lung function impairment, patients with asthma had significantly higher LTB₄ in the small airway or alveolar fraction than controls (5.57 pg/mL; IQR, 2.00–10.60 pg/mL; vs 2.00 pg/mL; IQR, 2.00–6.20 pg/mL; $P = .01$). LTB₄ concentrations of the large airways did not differ between patients with asthma and controls in this subgroup ($P = .41$). Other lung function parameters, such as total lung capacity, intrathoracic gas volume, residual volume/total lung capacity, or FeNO, did not correlate with LTB₄ concentrations of different fractions. There was no correlation of asthma control as measured by the ACT and LTB₄ levels in both fractions.

Discussion

To our knowledge, this is the first study to investigate LTB₄ concentrations in different fractions of EBC in asthma. For this purpose, we used a new noninvasive technique of fractionated EBC sampling to distinguish between the large airway and small airway or alveolar fraction of the lung. Our 2 principal findings are as follows. First, we found that LTB₄ is detectable and quantifiable in the small airway or alveolar fraction in pediatric asthma. Second, patients with asthma had significantly higher LTB₄ concentrations in the small airway or alveolar fraction than controls.

LTB₄ production in the asthmatic lung has been measured indirectly in bronchial lavage fluid, sputum, or EBC and has not yet been directly linked to a distinct lung compartment. It has been found to originate from mast cells, neutrophils, and alveolar macrophages. Localization in the asthmatic lung has been described in

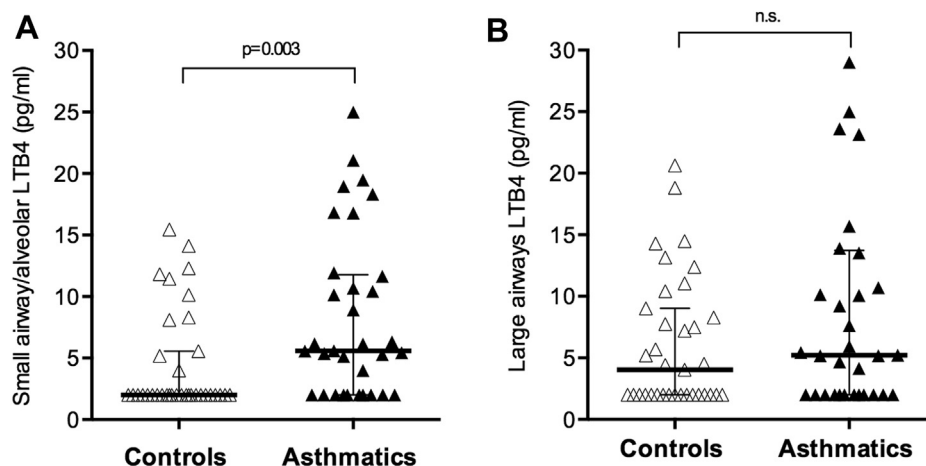


Figure 2. A, Leukotriene B₄ (LTB₄) concentrations measured by enzyme-linked immunosorbent assay in the small airway or alveolar fraction of exhaled breath condensate are significantly higher in children with asthma (5.58 pg/mL; interquartile range, 2.0–11.77 pg/mL; vs 2.0 pg/mL; interquartile range, 2.0–6.2 pg/mL; $P = .003$). B, No significant difference in the LTB₄ concentrations in the large airways of children with asthma and controls. n.s. indicates nonsignificant.

several studies and therefore might give indirect information about the predominant sites of LTB₄ production.^{5,6} For example, in a study examining mast cell distribution in central airway and trans-bronchial biopsy specimens, mast cells were significantly increased in the alveolar parenchyma of patients with uncontrolled, atopic asthma, hinting at the importance of peripheral production.^{11,19} This study aimed to describe relevant sites of LTB₄ production by measuring LTB₄ in 2 compartments of the lung using fractionated EBC sampling. Although the LTB₄ concentrations of both fractions are highly correlated, measuring small airway or alveolar LTB₄ may be a more precise marker to assess inflammatory processes in the asthmatic lung because LTB₄ concentrations were only elevated in the small airway or alveolar fraction of patients with asthma.

This bears relevance because measuring distal inflammatory markers is increasingly proposed to define asthma phenotypes and improve treatment monitoring. We and others found that the normal distribution pattern of other inflammatory markers usually exhibits higher values in the airways.^{3,4,20} This was found for nitric oxide through multiple flow analysis in children and adults with asthma, as well as by using in situ hybridization of lung biopsy

specimens for interleukin 4 and by measuring fractionated EBC for hydrogen peroxide. In the present study, a trend toward higher LTB₄ concentrations in the airway fraction could be observed, although this did not reach the required significance level. However, several studies also found that even though the alveolar levels of inflammatory markers are lower than the airway levels, they might be more important in characterizing the disease because they are correlated with the severity and symptoms.^{2,4,21} Puckett et al³ defined different distribution patterns of exhaled nitric oxide and therefore formed nitric oxide–associated inflammatory phenotypes of asthma. Compared with patients with elevated exhaled nitric oxide levels only in the airways, patients with elevated alveolar nitric oxide had worse asthma control independently whether the exhaled nitric oxide values in the airways were elevated or not. Our group reported a correlation of peripheral hydrogen peroxide concentrations and asthma control in a previous study.⁴ Here, a significant correlation for LTB₄ with asthma control, measured by the ACT, could not be shown, possibly because of our relatively well-controlled cohort. However, we measured significantly higher small airway or alveolar LTB₄ levels in children with a

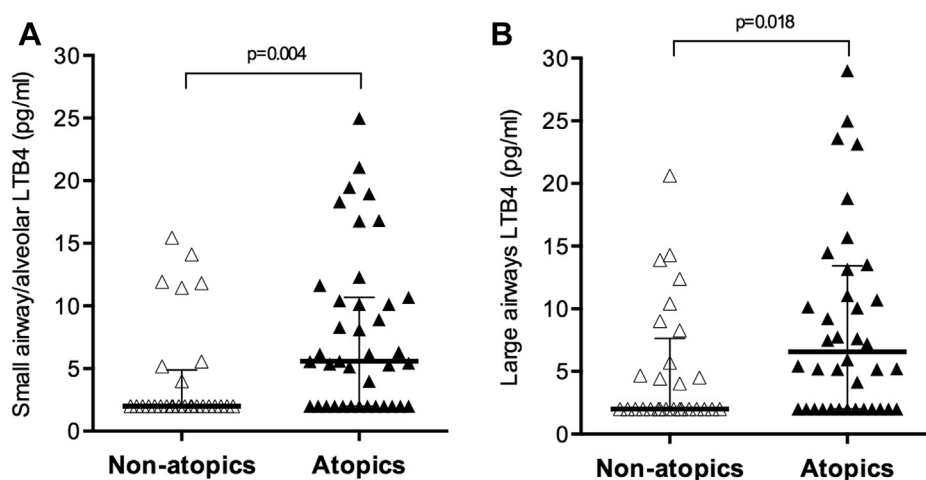


Figure 3. Atopic children have higher concentrations of leukotriene B₄ (LTB₄) than nonatopic children in the small airway or alveolar fraction (5.59 pg/mL; interquartile range [IQR], 2.00–10.68 pg/mL; vs 2.00 pg/mL; IQR, 2.00–4.88 pg/mL; $P = .004$) (A) and in the large airway fraction (6.58 pg/mL; IQR, 2.00–13.43 pg/mL; vs 2.00 pg/mL; IQR, 2.00–7.64 pg/mL; $P = .02$) (B).

pathologic z score for FEV₁ suggesting that alveolar LTB₄ concentrations may contribute to obstructive lung function testing. Children with pathologic z-scores for FEF_{25–75} exhibited increased LTB₄ levels not only in the small airway or alveolar fraction but also in the large airway fraction. Other typical peripheral lung function parameters, such as residual volume/total lung capacity, as well as suggested parameters such as intrathoracic gas volume and total lung capacity did not correlate with LTB₄ concentrations in our cohort.^{22,23} However, in children lung function parameters are mostly within normal limits and therefore not always conclusive.²⁴ Recently, technical advances have been made that improve the measurement of small airway function in patients with mild to moderate asthma, such as single-breath washout and impulse oscillometry. Studies using these techniques could reveal pathologic measurements in the small airways of children with uncontrolled asthma, but even patients with mild to moderate asthma exhibit abnormal acinar ventilation heterogeneity, suggesting the presence of small airway disease.^{25,26} With this in mind, it is even more important that in our cohort patients with asthma without pathologic z scores for FEV₁ and FEF_{25–75} still have increased LTB₄ values in the small airway or alveolar fraction compared with controls. This finding suggests that small airway or alveolar LTB₄ is a sensitive, noninvasive marker of small airways disease for children with asthma, even without lung function impairment.

Atopic patients have increased LTB₄ concentrations compared with nonatopic patients in EBC.²⁷ In the present study, we confirm these results in our cohort; children with atopic asthma have significantly higher LTB₄ concentrations in the small airway or alveolar fraction compared with nonatopic children with asthma. Even patients with atopic dermatitis without a lung disease exhibit increased LTB₄ concentration in EBC samples.²⁸ Decreased LTB₄ concentrations after corticosteroid and montelukast treatment hint to a role of LTB₄, especially in allergic, IgE-mediated asthma.^{6,9,10} Alveolar macrophages cultured from bronchoalveolar lavage samples of patients with asthma and controls revealed decreased LTB₄ concentrations after pretreatment with corticosteroids, but no difference was found in the suppression capabilities between controls and patients with nonsevere asthma.²⁹ We could not find a difference in LTB₄ concentrations between patients receiving and not receiving inhaled corticosteroid therapy, and there was no correlation of the LTB₄ concentration with inhaled corticosteroid dose. This finding might again be due to our cohort of patients with relatively well-controlled asthma and the reduced adherence of patients with well-controlled asthma. Patients included in this study had a mean ACT score of 23.

Fractionated EBC sampling emerges as a noninvasive research and diagnostic technique that might help to classify asthma phenotypes and improve therapy monitoring in the future.³⁰ In this study, we collected EBC using a capnograph-based method to determine the large airway and small airway or alveolar threshold. The initial separation is more precise for each patient using the plateau phase of the exhaled carbon dioxide concentration compared with a volume-based approach. However, the method is more time-consuming. Nevertheless, contamination of the small airway or alveolar fraction passing the larger airways might occur as a consequence of the nature of the technique. However, the technique is noninvasive, which is especially important in children and may be used repeatedly in asthma monitoring. The lack of correlation to FeNO values shows that a different type of inflammation or phenotype is measured. A potential weakness of this study is the missing use of a saliva trap because saliva is a source of LTB₄.³¹ However, differential concentrations of LTB₄ in both fractions of EBC point to the importance of LTB₄ measurements that originate from the airways and alveolar regions.

In summary, we found that LTB₄ concentrations are detectable in the small airway or alveolar fraction of EBC, where they were

significantly elevated compared with controls. This difference could not be seen in the large airway fraction in children with asthma. Furthermore, our data indicate an association between small airway or alveolar LTB₄ concentrations and obstructive lung function impairment. The significant elevation of LTB₄ in children with asthma with nonpathologic lung function may also be interpreted as a sign of its sensitivity as a noninvasive marker. Small airway or alveolar LTB₄ concentrations measured with the new technique of fractionated EBC sampling may therefore be a new marker of distal lung inflammation. Future long-term studies are needed to evaluate whether this technique is useful in asthma monitoring.

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