

# Markers of Airway Inflammation in the Exhaled Breath Condensate of Preschool Wheezers

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## ■ Abstract

*Background:* Leukotrienes (LT), isoprostanes, and nitrites/nitrates 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 LTB<sub>4</sub>, LTE<sub>4</sub>, 8-isoprostane, and nitrite/nitrate levels in the EBC of healthy and wheezing preschool children.

*Methods:* We included 21 healthy nonatopic children and 25 patients with recurrent wheezing episodes in a cross-sectional study. LTB<sub>4</sub>, LTE<sub>4</sub>, and 8-isoprostane concentrations were measured directly in EBC using a specific enzyme immunoassay; nitrite/nitrate concentrations were measured using a colorimetric assay.

*Results:* LTB<sub>4</sub> concentrations were higher in children with wheezing episodes than in healthy controls (76 pg/mL vs 20 pg/mL,  $P < .001$ ). LTE<sub>4</sub> was increased in children with wheezing episodes than in healthy controls (68 pg/mL vs 35 pg/mL,  $P < .001$ ). Nitrite concentrations were higher in children with wheezing episodes than in healthy controls (14 pg/mL vs 9.7 pg/mL,  $P < .03$ ). We found no differences in 8-isoprostane and nitrate concentrations between the patients and the healthy controls.

*Conclusions:* Our findings suggest that EBC is a suitable noninvasive method for the assessment of airway inflammation and oxidative stress in preschool children. Levels of LTB<sub>4</sub>, LTE<sub>4</sub>, and nitrites were higher in children with recurrent wheezing episodes than in healthy controls.

**Key words:** Preschool. Exhaled breath condensate. Isoprostane. Leukotriene. Nitrite. Nitrate. Wheezing.

## ■ Resumen

*Antecedentes:* Los leucotrienos (LT), isoprostanos y nitritos/nitratos, que son biomarcadores de la inflamación de las vías aéreas, y el estrés oxidativo, pueden ser detectados en el condensado del aire exhalado (CAE). El objetivo de este estudio fue evaluar el LTB<sub>4</sub>, LTE<sub>4</sub>, 8-isoprostano, nitritos y nitratos en el CAE de niños preescolares sanos y con sibilancias.

*Material y métodos:* Se realizó un estudio transversal en el que se incluyeron 21 niños sanos no atópicos y 25 pacientes con sibilancias recurrentes. El LTB<sub>4</sub>, LTE<sub>4</sub> y 8-isoprostano, se midieron en el CAE mediante ensayo inmunoenzimático, y los nitritos/nitratos mediante método colorimétrico.

*Resultados:* Las concentraciones de LTB<sub>4</sub> fueron mayores en los niños con episodios de sibilancias que en controles sanos (76 pg/ml vs 20 pg/ml,  $p < 0,001$ ); El LTE<sub>4</sub> se incrementó también en niños con episodios de sibilancias en comparación con niños sanos (68 pg/ml vs 35 pg/ml,  $p < 0,001$ ). Las concentraciones de nitritos fueron mayores en los niños con episodios de sibilancias que en los controles (14 pg/ml vs 9,7 pg/ml,  $p < 0,03$ ).

No hubo diferencias en las concentraciones de 8-isoprostano y nitratos entre el grupo de niños enfermos y el grupo control.

*Conclusiones:* Nuestros hallazgos sugieren que el CAE es un método no invasivo para la evaluación de la inflamación de las vías respiratorias y del estrés oxidativo en lactantes y niños en edad preescolar.

Las concentraciones de LTB<sub>4</sub>, LTE<sub>4</sub> y de nitritos, se incrementaron en niños con episodios recurrentes de sibilancias en comparación con los controles sanos

**Palabras clave:** Preescolares. Condensado del aire exhalado. Isoprostano. Leucotrieno. Nitrito. Nitrate. Sibilancias.

## Introduction

Asthma has become the most common chronic disease of childhood. It is a heterogeneous process that is characterized by airway obstruction and hyperresponsiveness, chronic airway inflammation, and oxidative stress [1,2].

In children aged less than 6 years, asthma has not been fully characterized. Symptoms such as recurrent wheezing and airway obstruction and narrowing are present during the first years of life [1], and the factors contributing to the subsequent development of asthma in these early wheezers are poorly understood. The most frequent cause of airway obstruction is viral infection [3,4]; however, studies performed with bronchoscopy and bronchoalveolar lavage (BAL) in young children with recurrent wheezing episodes revealed significantly increased levels of inflammatory cells and markers [5,6]. While BAL remains the gold standard for assessing airway inflammation, its invasiveness makes it unethical as a routine approach, particularly in children [5,7]. Sputum induction is less invasive, and findings for cells in sputum are similar to those of BAL, although it is particularly difficult to apply in children younger than 4-6 years [8].

Interest in the measurement of the components of exhaled breath condensate (EBC) as a noninvasive method is increasing. Collection of EBC by tidal breathing through a mouthpiece connected to a cooling unit is a simple and noninvasive technique that is relatively easy to apply in children older than 4-6 years.

The literature contains few reports on EBC collected from infants aged less than 6 years. In some, EBC was collected during sedated sleep [9-13].

Leukotrienes (LT) and isoprostanes are 2 of the components that can be detected in EBC. The LTs are a family of lipid mediators derived from arachidonic acid through the 5-lipoxygenase pathway that act as potent constrictors and proinflammatory mediators and play a role in the pathophysiology of asthma [14]. F<sub>2</sub>-isoprostanes are markers of oxidative stress that are synthesized from arachidonic acid by free radical-catalyzed lipid peroxidation. Their synthesis is independent of the action of cyclooxygenase. The most prevalent isoprostane in humans is 8-epi-PGF<sub>2</sub>- $\alpha$ , also known as 8-isoprostane [15].

Nitric oxide (NO) is a biological messenger. In aqueous solution, it reacts rapidly with reactive oxygen species to form stable oxides of nitrogen, which include nitrite (NO<sub>2</sub>) and nitrate (NO<sub>3</sub>) [15]. Increased levels of these markers have been found in asthmatic patients and in patients with viral and bacterial infections [16]. However, Baraldi et al [17] demonstrated that exhaled nitric oxide (eNO) levels were normal in a group of infants after a single wheezing episode due to viral infection.

Increased LT levels have been found in the EBC of asthmatic adults and children [18-20]. No studies have analyzed LTB<sub>4</sub> in the EBC of infants and children younger than 6 years, although increased levels of this marker have been reported in BAL samples [5].

Increased concentrations of 8-isoprostane have also been reported in the EBC of asthmatic adults and children, thus revealing an association with disease severity [20]. Three

studies have investigated this marker in EBC in preschool children [13,17,21], although only 1 of them recruited children aged less than 6 years. No studies to date have analyzed LTE<sub>4</sub> in the EBC of preschool children.

The aim of the present study was to assess LTB<sub>4</sub>, LTE<sub>4</sub>, 8-isoprostane, and nitrite/nitrate concentrations in the EBC of healthy and wheezing preschool children.

## Material and Methods

### Patients

The study population comprised 2 groups of children aged 8 months to 5 years: 21 healthy nonatopic children (controls) and 25 patients with a history of recurrent wheezing episodes (patients) (Table). The children were recruited from the Allergy Unit of Hospital General Universitario and from the Pediatric Pneumology Unit of Hospital Clínico Universitario, both in Valencia, Spain. No significant differences in age were recorded between the groups.

Table. Characteristics and Exhaled Breath Condensate Findings of the Study Participants<sup>a</sup>

	Healthy Controls	Recurrent Wheezers
No. (males)	21.0 (12.0)	25.0 (19.0)
Age, mo	37.5 (23.0-55.5)	36.0 (18.5-48.5)
LTB <sub>4</sub> , pg/mL	20.4 (13.9-26.1)	76.0 (41.8-90.9)
8-Isoprostane, pg/mL	112.2 (63.0-135.3)	112.6 (66.5-134.3)
Nitrites, $\mu$ M	9.7 (4.8-14.9)	14.0 (6.7-20.3)
Nitrates, $\mu$ M	5.2 (1.4-8.8)	4.9 (0.9-7.6)
LTE <sub>4</sub> , pg/mL	22.6 (16.5-31.3)	68.1 (29.8-85.8)

Abbreviations: LT, leukotriene.

<sup>a</sup>Values are expressed as median (interquartile range), unless otherwise specified.

Recurrent wheezing bronchitis was defined as at least 3 episodes of wheezing diagnosed by a pediatrician during the previous year. The most recent episode of wheezing was between 2 weeks and 3 months before inclusion. The patients were not taking regular medication other than  $\beta_2$ -agonists as needed for symptom relief.

The healthy controls were infants and children with a suspected but unconfirmed drug reaction recruited from the allergy unit and from relatives of the personnel of the allergy unit. They had no personal or family history of asthma or atopy or respiratory infections in the 4 weeks preceding the study. Atopy was confirmed using skin prick tests for common allergens.

Children were examined by a physician before undergoing skin prick tests and EBC collection.

Children were excluded from the study if they had received oral/inhaled corticosteroids or montelukast in the previous 4 weeks or nonsteroidal anti-inflammatory drugs during the previous 2 weeks.

The Ethics Committee of Hospital General Universitario approved the protocol, and informed consent was obtained from the parents of the children recruited in the study.

### Collection of EBC

EBC samples were collected using a specially designed condensing chamber (Anacon, Biostec). 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 facemask connected to the condenser for 30 minutes while watching a cartoon. A temperature of  $-8^{\circ}\text{C}$  inside the condensing chamber throughout collection ensured that samples were frozen immediately. The collected EBC samples were stored at  $-70^{\circ}\text{C}$  until measurement.

$\text{LTB}_4$ ,  $\text{LTE}_4$ , and 8-isoprostane were measured using a specific enzyme immunoassay kit (Cayman Chemical), and nitrite/nitrate levels were measured using a colorimetric assay based on the Griess reaction (Cayman Chemical).

This investigation was carried out following the American Thoracic Society/European Respiratory Society Task Force guidelines for oral sample collection. Since  $\text{NO}_x$  is present on laboratory surfaces, including glassware and pipette tips, we took special precautions to avoid contamination of the sample and used highly pure (distilled/de-ionized) water to rinse any material that came into contact with EBC, including devices used for collecting, processing, and assaying EBC immediately before use [22].

### Statistical Analysis

Given the asymmetric distribution of the biomarker concentrations, values were expressed as median (interquartile range). Groups were compared using the Mann-Whitney test. Correlations between biomarker concentrations were investigated using Spearman rank correlation analysis. Statistical significance was set at  $P < .05$ .

## Results

The results are summarized in Figure 1.

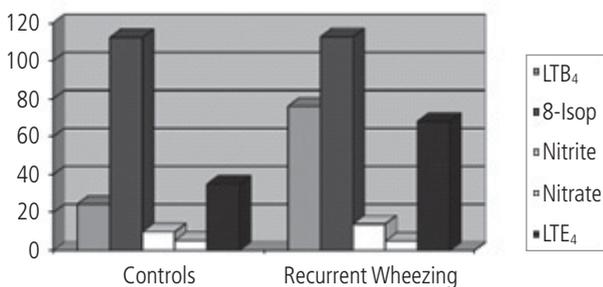


Figure 1.  $\text{LTB}_4$ ,  $\text{LTE}_4$ , nitrite, nitrate, and 8-isoprostane values in wheezing children and controls expressed as mean ( $P < .05$ ). LT indicates leukotriene; Isop, isoprostane.

### $\text{LTB}_4$

The level of  $\text{LTB}_4$  was significantly higher in patients than in healthy controls (Mann-Whitney, 14; mean [SD] difference,  $-53.5$  [11.6];  $P < .001$ ) (Figure 2).

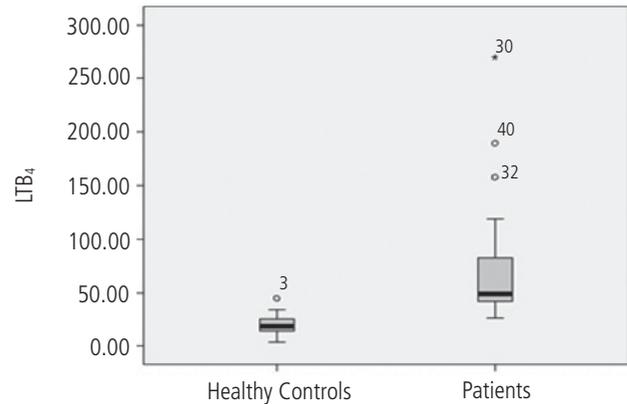


Figure 2.  $\text{LTB}_4$  values in wheezing children and controls. LT indicates leukotriene.

### $\text{LTE}_4$

The level of  $\text{LTE}_4$  was significantly higher in patients than in healthy controls (Mann-Whitney, 110; mean [SD] difference,  $-31.6$  [14.7];  $P < .001$ ) (Figure 3).

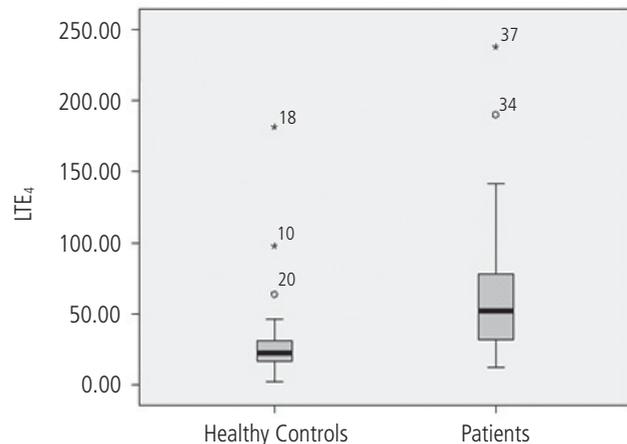


Figure 3.  $\text{LTE}_4$  values in wheezing children and controls. LT indicates leukotriene.

### Nitrites

Nitrite concentrations were higher in patients than in healthy controls (Mann-Whitney, 164; mean [SD] difference,  $-4.7$  [1.9];  $P < .03$ ) (Figure 4).

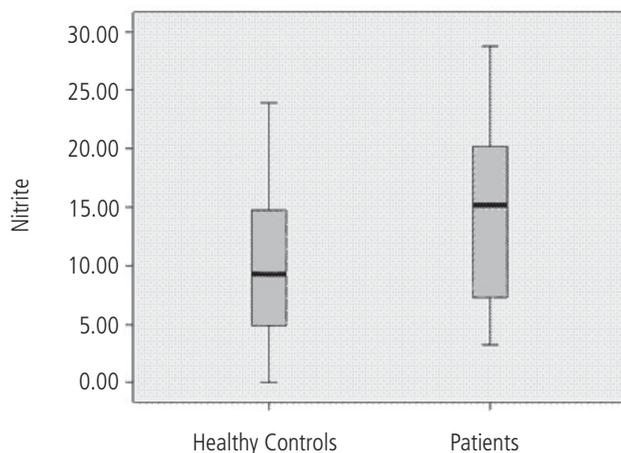


Figure 4. Nitrite values in wheezing children and controls.

### Nitrates

No differences were found in the nitrate concentration between patients and healthy controls (Mann-Whitney, 232; mean [SD] difference, 1.1 [1.7];  $P > .05$ ) (Figure 5).

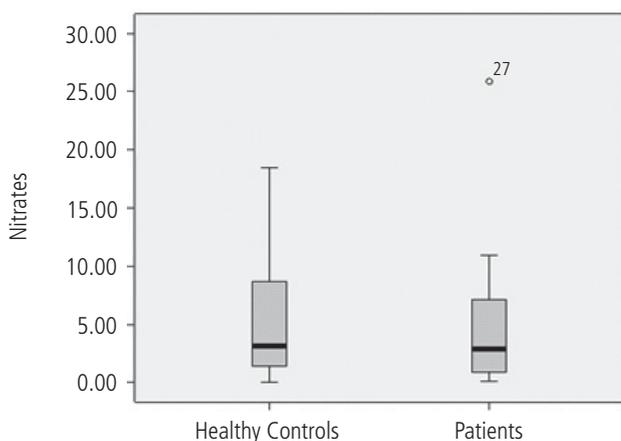


Figure 5. Nitrate values in wheezing children and controls.

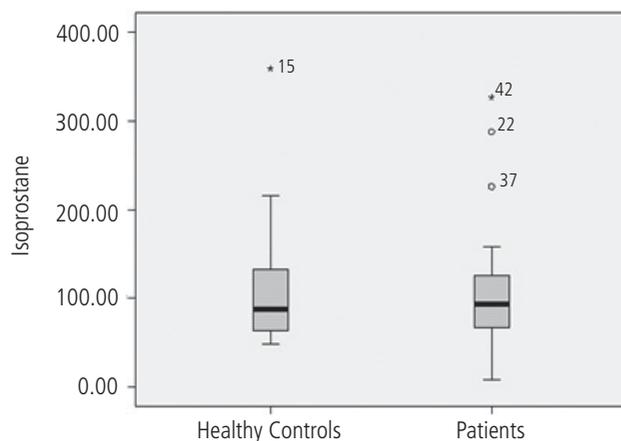


Figure 6. 8-Isoprostane values in wheezing children and controls.

### 8-Isoprostane

No differences were found in the 8-isoprostane concentration between patients and healthy controls (Mann-Whitney, 252; mean [SD] difference 5.1 [22.8];  $P > .05$ ) (Figure 6).

### Correlations

Correlations were analyzed in 3 groups: the overall study population, patients, and healthy controls. A significant correlation was observed between  $LTB_4$  and  $LTE_4$  concentrations in all 3 groups ( $r=0.59$ ,  $P < .001$ ). A significant correlation was found between  $LTB_4$  and  $LTE_4$  concentrations in the controls ( $r=0.5$ ,  $P < .01$ ) and in the patients ( $r=0.57$ ,  $P < .0001$ ).

A significant correlation was found between nitrites and  $LTE_4$  in all 3 groups ( $r=0.36$ ,  $P < .019$ ). A significant correlation was also found between nitrites and nitrates in the controls ( $r=0.47$ ,  $P < .036$ ) and in the patients ( $r=0.43$ ,  $P < .032$ ).

### Discussion

In our study, we observed that the biomarkers investigated were measurable in EBC without the need for sedation, even in infants as young as 8 months. The children cooperated, with the help of their parents, by breathing through a facemask while watching a cartoon or playing with their toys.

We show that children with recurrent wheezing episodes have higher levels of  $LTB_4$  in EBC than healthy children. No previous studies have analyzed  $LTB_4$  in the EBC of children aged less than 6 years, although increased levels of this marker have been reported in BAL samples [5].

$LTB_4$  exerts potent chemotactic action on airway neutrophils and acts as a mediator by inhibiting neutrophil apoptosis in vitro [23]. Neutrophils play an important role in the pathogenesis of asthma during exacerbations, in severe asthma, and in viral infections [7].

Using BAL, Krawiec et al [5] observed increased levels of  $LTB_4$  and  $LTE_4$  and the presence of neutrophils in wheezing infants, findings that are consistent with ours. Other authors [24] have reported increased levels of these markers in BAL samples from children with mild symptomatic asthma.

Our findings are also consistent with those of Csoma et al [20], who showed  $LTB_4$  to be higher in the EBC of children with mild and moderate-to-severe persistent asthma, than in patients with mild intermittent asthma and healthy individuals, although the children studied were aged more than 9 years. Our group confirmed these findings in children aged 6 to 14 years [25].

Most authors agree that  $LTB_4$  levels are higher in asthmatic children than in healthy children. Mondino et al [18] found  $LTB_4$  levels to be higher in patients taking and not taking corticosteroids than in a group of healthy children and a group of nonasthmatic atopic patients aged 8 years and over.

Cap et al [26] found  $LTB_4$  to be increased 1.6-fold in asthmatics aged 8 to 10 years and in adults, with respect to healthy individuals. In 2005, in children of the same age group, Montuschi et al [19] found  $LTB_4$  to be increased in asthmatic children not receiving corticosteroid treatment. In contrast, values were lower in nonasthmatic atopic children receiving corticosteroids.

In 2004, Bodini et al [27] also found higher LTB<sub>4</sub> levels in children aged more than 8 years exposed to the allergen than when exposure was suppressed.

Although no specific cutoff has been established for differentiating between LTB<sub>4</sub> levels in healthy and asthmatic individuals, evidence is growing of the usefulness of EBC in measuring bronchial inflammation and of the applicability of LTB<sub>4</sub> as an expression of neutrophil-mediated bronchial inflammation.

Consistent with findings for asthmatic children and adults, we found that increased LTB<sub>4</sub> in wheezers indicates airway inflammation at a very early age and can likely provide important information on severity and prognosis.

We also found LTE<sub>4</sub> to be higher in wheezing children than in healthy controls. A member of the cys-LT group, LTE<sub>4</sub> rises in patients with asthma. LTE<sub>4</sub> is even increased in adults with mild asthma [28].

No studies have investigated LTE<sub>4</sub> levels in children and preschool children, although our results are consistent with those of studies in older children. In a study of 48 children aged 7-14 years, Csoma et al [20] revealed significant differences between the cys-LT levels of asthmatic and healthy children, both in the mild asthma group and in the moderate-severe group. Another study in children and adults with mild and moderate-persistent asthma found that LTE<sub>4</sub> levels were higher than in healthy people [26]. Similar results were found for asthmatic children aged 8 to 14 years, especially in those with unstable asthma, as well as in atopic children with asthma, in comparison with atopic nonasthmatic children [18].

We also analyzed 8-isoprostane, a metabolite of arachidonic acid [29] and observed no differences between the values obtained in healthy individuals and in patients. This finding is consistent with those of Latzin et al [13], who observed that levels of 8-isoprostane were no higher in children older than 1 month with cystic fibrosis, recurrent wheezing, and acute lower respiratory tract infection.

Given that 8-isoprostane is a marker of oxidative stress, longer progression of inflammatory disease may be necessary for levels to increase, thus explaining the larger number of studies involving older children. In 2009, Cruz et al [30] demonstrated that 8-isoprostane levels increased with age in healthy adults and children. No similar studies have analyzed this biomarker only in children aged less than 7 years, although there could be an association between levels of the marker and disease duration. In our study, levels of 8-isoprostane were almost the same in both groups, possibly because of the short progression of disease in wheezing children.

However, some studies including children aged less than 6 years found an increase in levels of this biomarker. For example, in a study population of children aged 4 years and older, Baraldi et al [31] detected higher 8-isoprostane levels in asthmatics than in healthy children, although only during exacerbations. We are unable to draw comparisons with our sample, in which children were asymptomatic between episodes.

In the study of Shahid et al [21], conducted in children aged 2 to 18 years, values were also higher in asthmatics than in healthy children. However, as the authors did not perform an independent analysis of data in children aged less than 6 years, the results cannot be compared with ours.

In our group [25], 8-isoprostane levels in children older than 6 years increased in the group with more severe asthma. Therefore, 8-isoprostane could be associated with age, disease progress, and severity of asthma.

We found significant differences in nitrite levels, which were higher in patients than in healthy children. No differences were observed for nitrates. The only published study to investigate these markers in the EBC of infants was that of Formanek et al [32], who measured EBC values using the Griess reaction in children aged 3 years and older and found that nitrite values were higher in the asthmatic children than in the healthy ones.

These higher levels in wheezers could be related to the increased frequency of viral infections in this age group. Viral infections are associated with transient increases in exhaled NO concentrations [16], and infection by rhinovirus or respiratory syncytial virus induces expression of the enzyme nitric oxide synthase by airway epithelial cells [33], thereby increasing the metabolism of nitric oxide and generating nitrogen products, such as nitrites.

Nitrites could be also affected by oxidative reactions in the air or by contamination through contact with surfaces. We took special precautions with the EBC samples (see Material and Methods) in order to avoid contamination.

Given that some sample containers can leach out NO<sub>x</sub>, assays should be performed as soon as possible after collection, as was the case in our study. De-aeration with a CO<sub>2</sub>-free gas is recommended in the measurement of pH [22], although this marker was not investigated in the present study.

Nitrite values have been correlated with the fraction of exhaled NO in older children [34]. In our study, the child's age made it impossible to determine this parameter with the analyzer we used.

In our opinion, inflammatory markers could prove useful for the diagnosis of children with chronic airway inflammation and for establishing the most beneficial time to add or discontinue anti-inflammatory treatment. In addition, higher levels of these markers could indicate which children are likely to have asthma in the future.

Studies in older children have shown that LTB<sub>4</sub> is associated with neutrophilic inflammation and LTE<sub>4</sub> with eosinophilic inflammation [20,35]. However, in a study population of children aged less than 3 years, Bourgeois et al [7] showed that eosinophil levels were no higher in children with wheezing than in controls. Moreover, the number of neutrophils in children with wheezing was not correlated with bacterial or viral infection or atopic status [7].

As a secondary objective, we investigated differences between wheezing children whose prick test result to *Dermatophagoides* was positive and those whose result was negative; however, no significant differences in inflammatory markers were found, maybe because of the small number of children with positive prick test results (only 9). In our opinion, significant differences seem more likely with older children [36,37], a larger sample, and subgroups [37].

These markers remain unsuitable for daily practice until more robust data are available to corroborate our findings in preschool children. In addition, further studies are necessary to standardize the EBC technique and to define a cutoff for

differentiating between asthmatic/wheezing children and healthy children.

In conclusion, we demonstrated that EBC can be collected in nonsedated infants and that the method is feasible and safe in all age groups. Our initial results seem to confirm that inflammation is present in the airways of very young wheezing children, even when they are asymptomatic between episodes. In addition, determination of inflammatory mediators in EBC could prove useful for the diagnosis and management of wheezing children.

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