

# Exhaled breath condensate eicosanoids and sputum eosinophils in asthmatic children: A pilot study

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Cysteinyl leukotrienes (cys-LTs), LTB<sub>4</sub> and 8-isoprostane are increased in the exhaled breath condensate (EBC) from asthmatic patients. The aim of this study was to investigate whether the measurement of cys-LTs, LTB<sub>4</sub> and 8-isoprostane in EBC can reflect the level of airway inflammation assessed by induced sputum in asthmatic children sensitized to house dust mite (HDM) during natural avoidance of HDM allergens. Twelve children were evaluated at the time of admission (T0) and after 3 months of stay (T1) at the Istituto Pio XII (Misurina, Italian Dolomites 1756 m). Sputum eosinophil percentage and measurement of cys-LTs, LTB<sub>4</sub> and 8-isoprostanes in the breath condensate at T0 and T1 were evaluated. Eosinophil percentage in induced sputum was  $8.5 \pm 1.1\%$  at T0 and  $3.5 \pm 0.4\%$  at T1 ( $p = 0.011$ ). Neutrophil percentage in sputum was  $1.1 \pm 0.5\%$  at T0 and  $1.5 \pm 1.0\%$  at T1 (ns). Cys-LTs mean level was  $14.24 \pm 4.53$  pg/ml at T0 and  $4.65 \pm 0.68$  pg/ml at T1 ( $p = 0.0125$ ). LTB<sub>4</sub> level was  $2.36 \pm 0.19$  pg/ml at T0 and  $2.41 \pm 0.23$  pg/ml at T1 (ns). 8-Isoprostane level reduced from  $17.47 \pm 3.18$  pg/ml at T0 to  $7.36 \pm 3.26$  pg/ml at T1 ( $p = 0.003$ ). This study shows that exhaled cys-LTs and 8-isoprostane, as well as eosinophil percentage in induced sputum, are reduced after allergen avoidance in asthmatic children suggesting a potential application of EBC for the non-invasive evaluation of airway inflammation in asthma in allergic asthmatic children.

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Cysteinyl leukotrienes (cys-LTs), LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, have been described to play a pivotal role in the pathogenesis of asthma (1,2) including airway remodeling (3). Cys-LTs are synthesized primarily by mast cells and eosinophils (3), LTC<sub>4</sub> and LTE<sub>4</sub> levels are increased in urine and bronchoalveolar lavage (BAL) during early and late asthmatic response (4) as well as in sputum supernatants collected from mild asthmatics during late response to allergen (5). LTE<sub>4</sub> levels are increased after short periods of allergen exposure (6) or decreased after avoidance (7) in allergic asthmatic patients.

Isoprostanes are synthesized from arachidonic acid by free radical catalyzed lipid peroxidation. They have been described to represent markers of

oxidative stress (8) and suggested to have a pathogenetic role in asthma (9). The levels of 8-isoprostane have also been demonstrated to be increased in plasma (10), urine (11), and BAL (11) of asthmatic patients.

Recently, it has been demonstrated that levels of both cys-LTs and LTB<sub>4</sub> (12) as well as of 8-isoprostane are increased in the exhaled breath condensate (EBC) from adult asthmatic patients, and they have been suggested to represent a new non-invasive approach to the monitoring of airway inflammation in asthma (13).

The aim of this study was to investigate whether the measurement of cys-LTs, LTB<sub>4</sub> and 8-isoprostane in the EBC, as well as eosinophil percentage in induced sputum, can reflect the level of airway

Table 1. Individual data and patients' characteristics

| Patient no. | Sex (male/female) | Age (years) | Height (cm) | Weight (kg) | Forced expiratory volume in 1 s (% predicted) |
|-------------|-------------------|-------------|-------------|-------------|-----------------------------------------------|
| 1           | M                 | 13.8        | 177         | 84          | 107                                           |
| 2           | M                 | 9.5         | 136.5       | 29.8        | 97                                            |
| 3           | M                 | 12.9        | 166         | 49          | 110                                           |
| 4           | F                 | 14          | 154         | 47.2        | 103                                           |
| 5           | M                 | 13.6        | 157         | 44.5        | 115                                           |
| 6           | M                 | 12.4        | 156         | 45.5        | 92                                            |
| 7           | M                 | 9.11        | 149         | 42.5        | 92                                            |
| 8           | M                 | 8.2         | 132.5       | 28.5        | 92                                            |
| 9           | M                 | 15.7        | 157         | 44          | 99                                            |
| 10          | F                 | 14.1        | 152         | 79.5        | 115                                           |
| 11          | M                 | 13.11       | 161         | 47.5        | 109                                           |
| 12          | M                 | 9           | 134.5       | 36          | 112                                           |

inflammation in asthmatic children sensitized to house dust mite (HDM) during natural avoidance to HDM allergens at high altitude.

## Material and methods

### Subjects

Twelve HDM sensitized children (10 males, two females), age 8–15 years, with a history of mild bronchial asthma were evaluated at the residential house 'Istituto Pio XII' (Misurina, 1756 m, Italian Alps) (Table 1). All of the children had to be able to perform induced sputum test in order to be admitted to the study.

None of the patients had respiratory infections for at least 2 months before the beginning of the study and none of them was allergic to furred pets.

None of the children had received oral corticosteroids for at least 2 months before the study.

The study was approved by the Istituto Pio XII Ethics Committee and both children and their parents gave informed consent.

### Study design

The children had been living with their families for at least 3 months before admission to Istituto Pio XII.

Repeatedly performed assays failed to show detectable levels of group I HDM allergen in the residential house.

The first set of evaluations was performed in September (T0), when the children were admitted to the residential home, and the second set of evaluation was performed in December (T1), immediately before they left the Istituto Pio XII to spend Christmas holidays at sea level. This study was performed out of the pollen season in

order to avoid interference of outdoor allergens for those patients who were also sensitized to pollens.

At the time of admission to the residential house the children were receiving regular treatment with inhaled steroids, either fluticasone 100–200 mcg/day or budesonide 200 mcg/day. During the time of the study the clinical conditions allowed to gradually withhold inhaled corticosteroids in all of the children.

None of the children received oral steroid after admission to the residential house.

**Sputum induction.** Sputum was induced by inhalation of hypertonic saline solution, by a standardized method (14). Briefly, hypertonic saline solution was nebulized using an ultrasonic nebuliser (UN202, Medical Service Brescia, Italy), for four periods of 5 min each, for a total time of 20 min. Starting concentration was 4% NaCl delivered for a total of 10 min, which was followed by a 5% NaCl solution for further 10 min. Before hypertonic saline inhalation, all the patients were premedicated with inhaled salbutamol (two puffs, 200 mcg), to inhibit possible bronchoconstriction. The forced expiratory volume in 1 s (FEV<sub>1</sub>) was recorded before and 10 min after salbutamol and then every 5 min period during the saline inhalation. If FEV<sub>1</sub> fell by more than 20% of the post-bronchodilator value or troublesome symptoms occurred, nebulization was to be discontinued. After each period of nebulization, subjects were instructed to rinse their mouth and then to cough sputum into a Petri dish. In the laboratory, the Petri dish was placed against a dark background, making easier the identification of sputum portion in the watery saliva. Plugs arising from lower respiratory tract were selected by visual inspection, suspended in dithiotreitol for 30 min at 37°C and then centrifuged at 400 *g* for 10 min at room temperature. The cell pellets were washed twice in 1 ml 0.9% saline solution. The pellet was resuspended in 0.9% saline and the samples were transferred onto slides by cytocentrifugation (500 rpm for 5 min) with CYTOSPIN 3 (Shandon, Thermolectron Corp., Waltham, MA, USA). Slides were either fixed in alcohol 95° and then stained with May-Grunwald-Giemsa. Slides were coded to be counted blind to the study design. A differential cell count was obtained by counting at least 400 cells. The counts of eosinophils and neutrophils were expressed as mean percentages of total cells counted by two independent observers, excluding squamous epithelial cells to correct for salivary contamination of the samples.

**Exhaled breath condensate collection.** Exhaled breath condensate samples were collected in a condensing device formed by two glass chambers (Incofar Srl, Modena, Italy) (13). The inner glass chamber was cooled by means of ice and suspended in a larger glass chamber. The children, without nose clip, were instructed to tidally breathe by the mouth through a two-way non-rebreathing valve for 15 min. To minimize salivary contamination the two-way valve served as a saliva trap, with a 12 cm banded tube vertically positioned between the mouthpiece and the condensing device while the mouth of the subject remained at a lower position with respect to the inlet of the device. In addition, children were asked to periodically swallow their saliva. The collected EBC samples were then immediately stored in sterile tubes at  $-70^{\circ}\text{C}$ . 8-Isoprostane and cys-LTs ( $\text{LTC}_4/\text{D}_4/\text{E}_4$ ) concentrations in EBC were measured by specific enzyme immunoassay (EIA) kits (8-isoprostane,  $\text{LTB}_4$  Cayman Chemical, Milan, Italy;  $\text{LTC}_4/\text{D}_4/\text{E}_4$ , Amersham Pharmacia Biotech, Milan, Italy) as previously described (15–16). The method was modified to lower the detection limit (8-isoprostane 1 pg/ml, cys-LTs 4 pg/ml); for those samples below the lowest detection limits the data were expressed as half the detection limit (15).

**Lung function and NO measurement.** Lung function was measured ( $\text{FEV}_1$  was considered) by a Vitalograph Compact spirometer (Vitalograph Ltd, Buckingham, UK).

Fraction exhaled nitric oxide (FeNO) was measured by chemiluminescence analyser (Logan LR 2149, Rochester, Kent, UK), according to the 1999 American Thoracic Society (ATS) Guidelines and expressed in parts per billion (p.p.b.) (17).

#### Statistical analysis

As the data were not normally distributed, the differences of the inflammation markers (cys-LTs,

$\text{LTB}_4$ , 8-isoprostane, and sputum eosinophil cell count) between September (T0) and December (T1), were evaluated by Wilcoxon test. A p-value of  $<0.05$  was considered significant.

#### Results

All of the children completed the study and no adverse event was observed during the study period. At T1 it was not possible to collect adequate sputum sample in two children and in another one the dosage of soluble markers in EBC was not possible for technical reasons, i.e., inadequate amount of condensate to perform assays.

It was not possible to make any comparison on symptom score before and after admission to the residential house since the children were not known before T0; however, the clinical conditions allowed to gradually withheld inhaled corticosteroids in all of the children during the time of the study.

Eosinophil percentage in induced sputum was  $8.5 \pm 1.1\%$  at T0 and  $3.5 \pm 0.4\%$  at T1 ( $p = 0.011$ ).

Neutrophil percentage in sputum was  $1.1 \pm 0.5\%$  at T0 and  $1.5 \pm 1.0\%$  at T1 (ns).

Cys-LTs mean level was  $14.24 \pm 4.53$  pg/ml at T0 and  $4.65 \pm 0.68$  pg/ml at T1 ( $p = 0.0125$ ).  $\text{LTB}_4$  level did not change during the study period, being  $2.36 \pm 0.19$  pg/ml at T0 and  $2.41 \pm 0.23$  pg/ml at T1 (ns).

8-Isoprostane level reduced from  $17.47 \pm 3.18$  pg/ml at T0 to  $7.36 \pm 3.26$  pg/ml at T1 ( $p = 0.003$ ).

The individual data for eosinophil percentage in sputum samples, cys-LTs and isoprostane in EBC are plotted in Fig. 1 (panel a–d).

The measurement of FeNO at T0 was possible only in four subjects because of technical reasons, i.e., failure in analyser calibration. In these four subjects the mean levels of FeNO were 25.5 p.p.b. at T0 and 18.1 p.p.b. at T1.

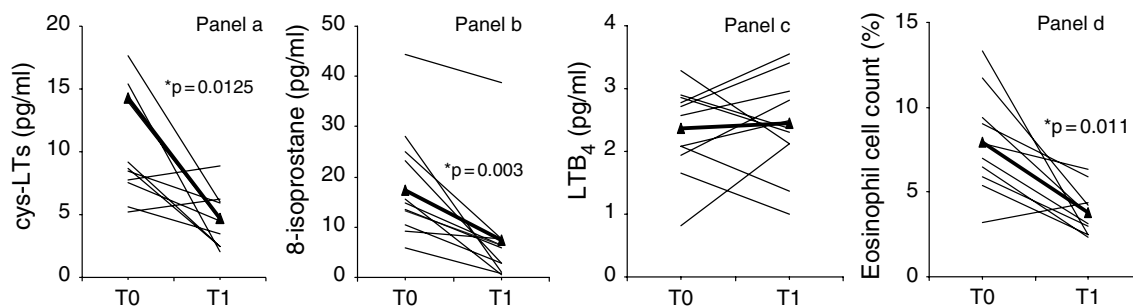


Fig. 1. Values of cysteinyl-leukotrienes (cys-LTs; panel a), 8-isoprostane (panel b),  $\text{LTB}_4$  (panel c) and sputum eosinophil cell count (%) (panel d) before (T0) and after (T1) 3 months of antigene avoidance. \* $p < 0.05$  compared with T0. — values single patient, — geometric mean values.

## Discussion

House dust mite avoidance can be effectively achieved during periods of stay in high altitude clinics (7), where effective allergen avoidance can be obtained because of the extremely low number of HDM, mainly because of the low level of indoor humidity in the mountain environment (18).

Previous studies have demonstrated an improvement in lung function and a reduction in bronchial hyper-responsiveness (BHR) (19, 20) in mite-allergic asthmatic children resident at high altitude clinics.

In addition, a significant reduction in airway inflammation has been documented by a reduction of both cell phase (21) and exhaled markers of inflammation (22) despite no association of inflammation markers with current symptoms has been reported (23).

This was a pilot study to evaluate whether the levels of cys-LTs and 8-isoprostanes in EBC from asthmatic children were influenced by the allergen avoidance in the mountain environment, and, as a consequence whether the evaluation of these markers in EBC could represent a valid method to monitor airway inflammation in asthmatic children participating to allergen avoidance programs. Despite the 'gold standard' for measuring airway inflammation in asthma is represented by fiberoptic bronchial biopsies, this is an invasive procedure which cannot be repeated often and it is unsuitable in children (24). For this reasons, as the method of induced sputum for cellular and fluid-phase markers of inflammation has provided a relatively non-invasive, valid and repeatable method of assessing airway inflammation, although with a lower success rate in children, in this study we used this method to evaluate the level of airway inflammation.

As expected on the basis of previous studies (21) a significant reduction in eosinophil airway infiltration has been observed after 3 months of stay at high altitude, despite stopping steroid treatment, which could have opposed the beneficial effect of HDM avoidance, thus confirming a reduction in airway inflammation in our group of asthmatic children.

Similarly, we observed a significant reduction in cys-LTs and in 8-isoprostane levels in the EBC. Montuschi et al. (12) demonstrated that cys-LTs and LTB<sub>4</sub> are increased in the EBC from asthmatic patients not treated with corticosteroids. More recently it has been shown that exhaled cys-LTs and LTB<sub>4</sub> are significantly increased in the EBC from children with mild persistent and moderate asthma but not in those

with mild intermittent asthma (25). Therefore, the authors concluded that EBC may be useful in assessing airway inflammation in asthmatic children.

The present study failed to show a significant reduction in LTB<sub>4</sub> in our group of mild allergic asthmatic children. A reduction in LTB<sub>4</sub>, a potent chemoattractant of neutrophils, was however, not expected as airway inflammation in our study population was mainly eosinophilic, as expected in children with allergic mild-moderate asthma (26, 27). This hypothesis is further supported by the observation that the percentage of neutrophils in sputum in our study population was unchanged after the period of mite avoidance. A more direct effect on LTB<sub>4</sub> could be hypothesized in subjects with asthma related to environmental exposure to bacterial endotoxin, particulate air pollution, ozone or viral infections (27, 28), which was not really the case in our study population.

An inverse correlation was previously demonstrated between exhaled cys-LTs and LTB<sub>4</sub> in patients with mild persistent asthma (25). In our study population we failed to observe any correlation between the investigated parameters, possibly because of the low number of subjects. However, the results of this study suggest that cys-LTs reduction during the period of avoidance were concordant with a reduction in sputum eosinophil percentage, whereas, that the lack of modification in LTB<sub>4</sub> was consistent with the unchanged percentage in sputum neutrophils.

In the present study, the baseline level of LTB<sub>4</sub> was rather low as compared with previous observations (25). However, we were more interested in the differences between the two sampling time rather than the absolute concentrations in the samples.

More elevated levels of LTB<sub>4</sub> and neutrophil inflammation can be typically observed in more severe asthmatic children (29, 30), with a possible relationship to steroid treatment. In fact, as suggested in the recent study by Csoma (25), elevation in LTB<sub>4</sub> levels in EBC could be, at least in part, a consequence of corticosteroid treatment. In the above referred study the authors convincingly speculated about the possibility that steroid can elevate the levels of LTB<sub>4</sub> because of their action on neutrophil inflammation, which has been observed during asthma exacerbation and in more severe disease. In contrast cys-LTs can prolong eosinophil survival with a consequent further production of cys-LTs and eosinophil recruitment (31). The children admitted to the present study were mild asthmatics clinically

well controlled receiving low doses of inhaled steroids at the time of admission. From the small number of subjects participating to our pilot study it is not possible to draw any conclusion regarding the impact of steroid treatment at the time of admission and withdrawing in the following weeks. However, it is tempting to speculate that the treatment with inhaled steroids can have contrary effect in the modulation of neutrophil and eosinophil inflammation, and, as a consequence, on the levels of exhaled LTs.

Fractional exhaled nitric oxide concentrations have been reported to be particularly high in atopic asthmatic children sensitized to HDM allergen (32). In the study by Csoma and coworkers (25), no correlation was found between cys-LTs and exhaled NO (FeNO). In the present study, the measurement of FeNO at T0 was possible only in four subjects because of technical reasons, i.e., failure in analyser calibration. Despite in these four subjects it was observed a trend to a reduction in FeNO levels during the stay in the mountain environment, no conclusion can be drawn regarding the possible relationship between FeNO and either exhaled markers of inflammation or sputum eosinophil percentage in this study.

Unfortunately, in this study it was not possible to include a control group, since no healthy child is spending 3 months in the residential house Istituto Pio XII and the number of subjects is low because of the necessity to enroll only those who were able to perform sputum induction. Despite these factors may in part limit the interpretation of the results, the present study suggest that exhaled cys-LTs and 8-isoprostane levels as well as eosinophil airway inflammation are reduced after a 3-month stay in an Alpine environment.

In conclusion, these results are in favor for a potential application of the measurement of exhaled cys-LTs and 8-isoprostane in EBC for the non-invasive evaluation of airway inflammation in asthma in allergic asthmatic children. However, further studies are required in order to standardize the procedure of EBC collection and to validate the methods for the measurement of mediators in the EBC fluid before it can be proposed for more extensive practical application.

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