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# Eicosanoids in exhaled breath condensate and bronchoalveolar lavage fluid of patients with primary lung cancer

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**Abstract**. Although eicosanoids are involved in lung carcinogenesis they were poorly investigated in exhaled breath condensate (EBC) and bronchoalveolar lavage fluid (BALf) in patients with primary lung cancer. In this study 17 patients with diagnosed non-small cell lung cancer, 10 healthy smokers and 12 healthy nonsmokers were included. The levels of cys-LTs, 8-isoprostane, LTB4 and PGE2 were measured before any treatment in the EBC of all patients and in BALf of patients with lung cancer by enzyme linked immunosorbent assay. 8-isoprostane, LTB4, cys-LTs and PGE2 were detectable in the EBC and BALf. There were no significant differences between healthy smokers and nonsmokers in concentrations of all measured mediators. Compared with both healthy controls, patients with diagnosed lung cancer displayed higher concentrations of cys-LTs ($p < 0.05$) and LTB4 ($p < 0.05$) in EBC. In patients with lung cancer, the mean concentrations of all measured mediators were significantly higher in BALf compared with EBC and there was a significant, positive correlation between concentration of cys-LTs, $LTB_4$ and 8-isoprostane in BALf and their concentrations in the EBC ($r = 0.64$, $p < 0.05$, $r = 0.59$, $p < 0.05$, $r = 0.53$, $p < 0.05$ respectively). Since cys-LT, LTB4 and 8-isoprostane concentrations in EBC from patients with lung cancer reflect their concentrations in BALf, they may serve as a possible non-invasive method to monitor the disease and to assess the effectiveness of therapy.

Keywords: Exhaled breath condensate, broncholaveolar lavage, lung cancer, leukotrienes, prostaglandins, early detection

## 1. Introduction

One problem with the monitoring of patients with non-small cell lung cancer (NSCLC) is the lack of specific markers that could reflect disease severity and progression. Classifications, which describe the extent of cancer spread and grading systems, are complicated and not always effective in monitoring a patient's recovery. Furthermore, there are no methods that could be used in ambulatory to predict early cancer recurrence. The collection of exhaled breath condensate (EBC) is an easy to perform, non-invasive and repeatable method that samples volatile and non-volatile substances directly from the respiratory tract [1,2]. The method offered a new insight into the pathology of airways inflammation [1,3–5] however, in lung cancer the usefulness of EBC was poorly investigated.

Since in the lung cancer microenvironment, cyclooxygenase-2 (COX-2) and 5-lipooxygenase (5-LOX) are over-expressed [6,7] and oxidative stress is increased [8], we hypothesized that the end products of enzymatic (prostaglandin E2: PGE2, leukotriene B4: LTB4 and cysteinyl leukotrienes: cys-LTs) and nonenzymatic (8-isoprostane) metabolism of arachidonic acid could be detectable in EBC collected from patients with lung cancer.

The aim of this study was to assess the levels of cys-LTs, PGE2, LTB4 and 8-isoprostane in the EBC of patients with primary lung cancer and to compare with those detected in the bronchoalveolar lavage fluid (BALf) of patients diagnosed with NSCLC and those detected in EBC of healthy control. Furthermore, we wanted to investigate the relationship between levels of cys-LTs, PGE2, LTB4 and 8-isoprostane in EBC and

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their concentrations in BALf in patients suffering from primary lung cancer.

## 2. Methods

This study was approved by the Ethics Committee of the Medical University of Lodz and all subjects provided informed consent before participating. The characteristics of the study population are presented in Table 1. In 17 included patients diagnose of lung cancer was based on the results of biopsies performed in the hospital. The histological type of the tumor was restricted to the NSCLC (planoepithelial cancer, stage I and II). All patients were considered as metastatic free individuals on the basis of imaging studies performed during routine diagnostic and before receiving any anticancer treatment. Furthermore, all patients with diagnosed cancer had peripheral lesions in the lung, allowing the bronchoalveolar lavage performance at the lesion site.

In all patients EBC was collected just prior to bronchoscopy. Patients were allowed to use short-acting inhaled $\beta_2$-agonists but steroids were prohibited for at least 4 weeks prior to sample collection. Exclusion criteria included: respiratory tract infection for at least 4 weeks preceding the samples collection, tuberculosis, severe heart failure, uncontrolled hypertension, autoimmunologic disease. Patients should have had at least 1 L of forced expiratory volume in one second (FEV1) on the day of bronchoscopy. 10 healthy smokers and 12 healthy nonsmokers were included as a control group and underwent spirometry and the EBC (due to the difficulty of obtaining consent from the Ethics Committee of Medical University of Lodz to perform bronchoscopy in healthy individuals).

## 3. Collection of BALf

Bronchoalveolar lavage was performed during the bronchoscopy according to international standards [9]. Briefly, patients were topically anesthetized with lidocaine spray applied to the nostril and the pharynx. A flexible bronchoscope (Pentax, Tokyo, Japan) was wedged to the bronchus supplying segment with the tumor. Next 100 ml of sterile, isotonic NaCl was instilled into the segment and immediately aspirated by gentle suction. The mean recovery of BALf was $52.9 \pm 6.9\%$. BALf was collected in sterile tubes, kept on ice and processed immediately in the laboratory. There BALf was pooled, passed through a sterile nylon filter (Becton Dickinson, NJ, USA) and centrifuged (10 min, x 300, room temperature). Supernatant was collected for further analysis, whereas cell viability was performed by tryptan blue exclusion. The cells were counted under a light microscope and numbers of neutrophils, macrophages, lymphocytes and eosinophils were presented as a percentage of total cell count.

Table 1
Study population

| | Lung cancer | Control (smokers) | Control (nonsmokers) |
|---|---|---|---|
| Number of patients | 17 | 10 | 12 |
| Age (year) | $54.9 \pm 12.0$ | $49.9 \pm 15.2$ | $37.8 \pm 13.1$ |
| Sex (M:F) | 9:8 | 5:5 | 7:5 |
| FEV 1 (% of predicted value) | $77.4 \pm 9.4$ | $88.8 \pm 7.8$ | $95.6 \pm 8.9$ |
| BALf recovery (%) | $52.9 \pm 6.9$ | ND | ND |
| EBC volume (ml) | $1.57 \pm 0.2$ | $1.78 \pm 0.4$ | $1.77 \pm 0.3$ |
| Smoking history (years) | $16.3 \pm 4.7$ | $13.1 \pm 2.7$ | 0 |
| Packyears | $20.3 \pm 6.2$ | $16.1 \pm 3.2$ | 0 |
| Ex smokers (n) | 6 | 2 | 0 |
| Current smokers (n) | 11 | 8 | 0 |

Values given as the mean $\pm$ SD, ND – not done, n number of patients.

### 3.1. Exhaled breath condensate collection

In all patients EBC was collected prior to the bronchoscopy. Patients were instructed to breathe at a normal frequency and tidal volume through a mouthpiece connected to the sampling tube for 10–15 minutes with the respiratory rate between 15–20 breaths/min. Patients wore a nose clip and were asked to rinse their mouth with distilled water before and after the procedure to reduce evaporation of eicosanoids from the saliva and nasal spaces. The condensate was collected by a commercially available condenser (EcoScreen, Jaeger, Germany). Samples were tested for salivary contamination by the determination of amylase activity (Sigma-Aldrich, Poznan, Poland). Approximately $1.57 \pm 0.2$ ml, $1.78 \pm 0.4$ ml and $1.77 \pm 0.3$ ml of condensate was collected in patients with diagnosed lung cancer, healthy smokers and non-smoking subjects respectively and immediately stored up to 4 weeks at $-80^\circ$ C for further analysis.

### 3.2. Quantification of eicosanoids in EBC and BALf

#### 3.2.1. Leukotrienes

The EBC and BALf concentration of cysteinyl-leukotrienes (Cys-LTs) was examined by an enzyme

Table 2
Concentrations of cys-LTs, LTB4, PGE2 and 8-isoprostane in BALf and EBC of patients with lung cancer and healthy controls (in pg/ml)

| | cys-LTs | | $LTB_4$ | | $PGE_2$ | | 8-isoprostane | |
|---|---|---|---|---|---|---|---|---|
| | BALf | EBC | BALf | EBC | BALf | EBC | BALf | EBC |
| Healthy smokers | N.D. | 16.88 ± 5.3 (6.91) [2.5; 34.93] | N.D. | 16.02 ± 4.4 (12.5) [8.67; 20.07] | N.D. | 16.6 ± 4.1 (14.99) [2.95; 28.66] | N.D. | 15.06 ± 5.1 (10.1) [5.96; 20.72] |
| Healthy nonsmokers | N.D. | 13.73 ± 4.6 (6.8) [2.5; 25.07] | N.D. | 12.52 ± 2.84 (10.95) [3.72; 14.65] | N.D. | 15.63 ± 3.85 (12.37) [2.5; 28.28] | N.D. | 12.29 ± 3.1 (10.79) [2.5; 23.9] |
| Lung tumours | 39.2 ± 7.6 (36.8) [18.45; 48.54] | 23.4 ± 2.7* | 83.8 ± 14.7 | 23.8 ± 2.9* | 27.6 ± 5.8 (17.5) [7.5; 54.5] | 17.7 ± 2.4 (17.5) [7.5; 24.74] | 61.0 ± 11.4 | 18.2 ± 3.0 |

Values are presented as mean ± SEM, and (median) [25th; 75th percentile]. N.D. – not done, * $p < 0.05$ (vs healthy smokers and healthy nonsmokers).

immunoassay (EIA) kit (Cayman Chemical, MI, USA) as previously described [10]. $LTB_4$ was measured by an EIA kit (Cayman Chemical, MI, USA). The antiserum used in this assay has 100% cross-reactivity with $LTB_4$, 39% with 6-trans $LTB_4$, and $< 0.01\%$ with $LTC_4$, $LTE_4$, $LTD_4$, and $LTF_4$. The minimum detectable concentrations were: 13 pg/ml for cys-LTs and 4.43 pg/ml for $LTB_4$ [11].

*3.2.2. 8-isoprostane*

8-isoprostane concentration in EBC and BALf was measured by the EIA kit (Cayman Chemical, MI, USA) as previously described [12]. The antiserum used in this assay has 100% cross-reactivity with 8-isoprostane, 0.77% with prostaglandin F1, 0.66% with prostaglandin F3, 0.31% with prostaglandin E1. The detection limit of the assay was 5 pg/ml.

*3.2.3. $PGE_2$*

Prostaglandin $E_2$ concentration in EBC and BALf was measured by the EIA kit (Cayman Chemical, MI, USA). The antiserum used in this assay has 100% cross-reactivity with $PGE_2$, 43% with $PGE_3$, 18.7% with $PGE_1$, 0.1% each with $PGF_{2\alpha}$, $PGA_1$, $PGA_2$. The minimum detection limit was 1.5 pg/ml.

*3.2.4. Lung function test*

Pulmonary function parameters were measured before the bronchoscopy, with a computer-assisted spirometer (Lung Test 1000, Mes Dymek, Poland) according to standardized guidelines and best value of three maneuvers was expressed as a percentage of the predicted normal value.

*3.2.5. Statistical analysis*

Mean ± standard error of the mean (SEM) was provided for normally distributed data whereas median values with 25th and 75th percentiles were also presented when the data were not normally distributed. A Mann-Whitney test was used to compare groups and correlations between variables were evaluated by Spearman's test. Significance was defined as a value of $p < 0.05$ (GraphPad Prism 5, San Diego, CA).

## 4. Results

Three of NSCLS patients, two of healthy smokers and 6 of non-smoking healthy had 8 isoprostane below the detection limit. For LTB4 two of NSCLS patients, two of healthy smokers and three of non-smoking healthy had concentrations below the detection limit whereas for cys-LTs concentrations below the detection limit were in two of NSCLS patients, six of healthy smokers and five of non-smoking healthy.

In patients with diagnosed lung cancer there were higher concentrations of cys-LTs ($p < 0.05$) and LTB4 ($p < 0.05$) in EBC compared to both healthy controls (Table 2). Furthermore concentrations of $LTB_4$, 8-isoprostane and cys-LTs, were significantly higher in BALf compared to EBC ($p < 0.05$, $p < 0.001$, $p < 0.01$ respectively). Mean levels of PGE2 were higher in BALf when compared to EBC however, the difference was not significant ($p > 0.05$) (Table 2, Fig. 1). In patients with lung cancer concentration of LTB4 was highest both in BALf and EBC and the concentration of PGE2 was lowest both in BALf and EBC when compared with other mediators (Table 2, Fig. 1).

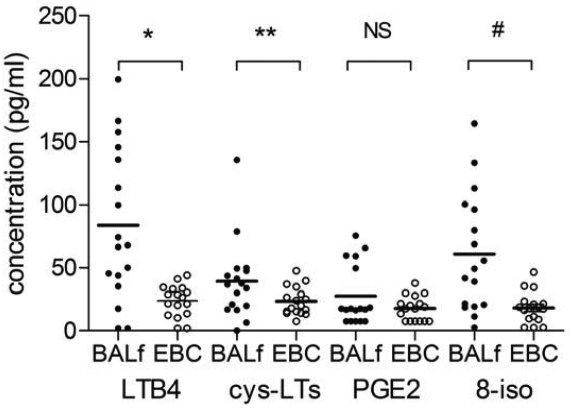


Fig. 1. Comparison of LTB4, cys-LTs, PGE-2 and 8-isoprostane in the BALf and EBC of patients with NSCLC. * $p < 0.001$, ** $p < 0.05$, # $p < 0.01$, NS – not significant.

Table 3
Correlation between mediators in EBC or BALf with BALf cytology in patients with NSCLC

| | | Correlations A with B | |
|---|---|---|---|
| Variable A | Variable B | r | p value |
| cys-LTs in EBC | macrophages in BALf | −0.67 | 0.003 |
| cys-LTs in BALf | basophils in BALf | −0.49 | 0.04 |
| PGE2 in BALf | eosinophils in BALf | −0.6 | 0.01 |
| PGE2 in BALf | basophils in BALf | −0.56 | 0.02 |

There was a strong positive correlation between both cys-LTs, $LTB_4$ and 8-isoprostane in BALf and those measured in the EBC ($r = 0.64$, $p < 0.05$, $r = 0.59$, $p < 0.05$, $r = 0.53$ $p < 0.05$ respectively) (Fig. 2) whereas correlation for PGE2 was not significant ($r = 0.47$, $p > 0.05$). We did not find correlations between the values of FEV1 and concentrations of eicosanoids both in EBC and BALf.

The cellular profile of BALf in patients with diagnosed lung cancer was as follows: macrophages 85.2 ± 1.8%, lymphocytes 9.8 ± 1.8%, neutrophils 1.8 ± 0.5%, monocytes 0.9 ± 0.3%, eosinophils 1.1 ± 0.3%, basophils 0.6 ± 0.2%, epithelium 0.3 ± 0.1%, vitality 98.3 ± 0.2%. There were some correlations between cell counts in BALf and mediators measured in both BALf and EBC. Briefly, the values of PGE2 in the BALf of patients with lung cancer presented a significant negative correlation with BALf basophils and eosinophils levels, cys-LTs in the BALf presented a significant negative correlation with BALf basophils levels whereas cys-LTs in EBC correlated negatively and significantly with BALf macrophages count (Table 3).

Although levels of LTB4, cys-LTs, PGE2 and 8-isoprostane were generally lower in EBC of healthy nonsmokers no statistical differences were found between the healthy smoking and nonsmoking subjects (Table 2).

## 5. Discussion

Our study demonstrates that 8-isoprostane, LTB4, cys-LTs and PGE2 are detectable in EBC and their concentrations are significantly lower than those in BALf in patients with diagnosed NSCLC. The concentrations of LTB4 and cys-LTs in breath condensate of these patients were higher than the concentrations measured in EBC of healthy, age-matched smokers and healthy nonsmokers. We demonstrated, for the first time, significant, positive correlations between levels of exhaled cys-LTs, LTB4 and 8-isoprostane and those of BALf and the lack of correlation between levels of these mediators in BALf and EBC and lung function parameters.

Breath condensate may provides information about pulmonary inflammation and oxidative stress. Collection of EBC has several advantages over the traditional sampling from the airways with BAL. It is easy to perform, non-invasive, repeatable, standardized [13] and collects metabolites originating locally in the airways and lungs [1,2]. Various gases and non-volatile compounds have been detected and studied in the EBC that may reflect the concentrations within the extracellular epithelial lining fluid in the airways. Biomarkers found in EBC were used to monitor asthma [1,2], COPD [1] and other inflammatory diseases [4,14] while in patients with lung cancer were poorly investigated.

It was demonstrated that lung carcinogenesis is associated with overexpression of COX-2 and 5-LOX [6, 7,15] and enhanced oxidative stress [16]. Their end products (LTB4, cys-LTs, PGE2, 8-isoprostane) are increased in the airway lumen of patients with lung cancer and have been implicated in peritumoral inflammation [14,16–19], tumor development [7,20–22], angiogenesis [23,24] and suppressesion of host defence mechanisms [20,24].

In patients with diagnosed lung cancer levels of LTB4 were elevated in EBC [11] and pleural effusion [25] whereas 8-isoprostane was found in EBC and serum [16] and PGE2 was measured in EBC [22].

Similarly, we have demonstrated that the levels of 8-isoprostane, LTB4 and cys-LTs are detectable in EBC. To validate the data obtained from EBC, BALf has been used in our study. Since both primary lung cancer and pulmonary metastases from other organs increase eicosanoids concentration in BALf [22] we included

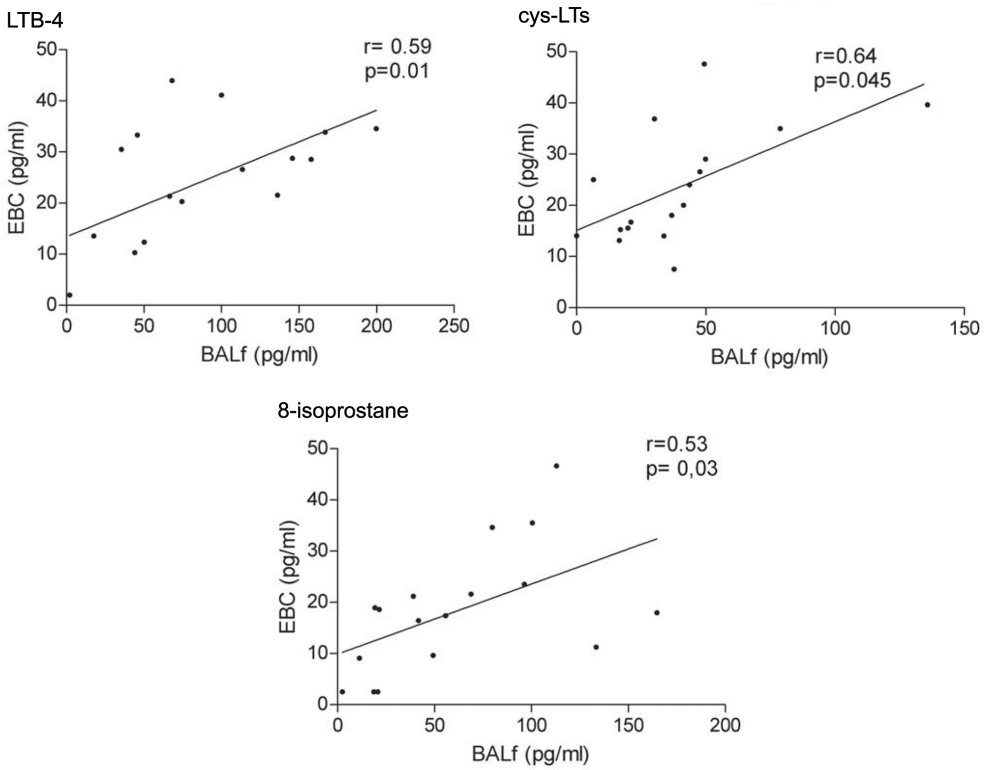


Fig. 2. Positive correlation between LTB4, cys-LTs and 8-isoprostane levels in BALf and EBC of patients with NSCLC.

patients with primary lung cancer without extrathoracic manifestations and we excluded all patients with extrathoracic malignancies that could give pulmonary metastases. Furthermore, the patients' age [19] and smoking status [26] may influence the concentrations of lipid mediators thus to eliminate the bias we have included age-matched healthy smokers and healthy, nonsmoking subjects as a control groups. In this way lipid mediators found in BALf and EBC reflected mainly its production within the lung by neoplasmatic and inflammatory milieu.

As we have demonstrated previously, the levels of PGE2 and cys-LTs were higher in EBC of patients with lung cancer when we compared with patients with noneoplasmatic diseases (COPD, chronic cough, sarcoidosis) and levels of LTB4 were higher than those observed in patients with chronic cough and sarcoidosis [27]. In this study, levels of cys-LTs and LTB4 in EBC were higher than in healthy. The explanation of this phenomenon could be twofold. Lung carcinogenesis may induce local inflammation that recruits inflammatory cells capable of releasing of reactive oxygene species and eicosanoids. However, cancer cells may induce oxidative stress and overexpress oxygenases synthesizing lipids mediators. In this study, similarly to other authors [1,28] we did not find a significant positive correlation between the levels of eicosanoids with number of macrophages and neutrophils in BALf. Moreover, we found significant negative correlation between PGE2 and macrophages count in BALf which constitute a main source of these mediators. Therefore, similarly to other investigators [1,28] we had a problem ascertaining the cellular source of mediators. However, if cancer cells are able to secrete lipids mediators [21] and there was no positive correlation between cells count and levels of lipids mediators in BALf and EBC, we may come to the plausible conclusion that tumor cells were the main source of these mediators.

Furthermore, we have demonstrated, for the first time, that concentrations of LTB4, cys-Lts and 8-isporostane in EBC correlated positively with that in BALf. BALf is a sample from only a portion of the lung and does not capture mediators from the airways, whereas EBC represents a whole lung sample and contains mediators both from the affected area of the lung and the rest of respiratory truct. Therefore, the concentration of lipids mediators is the highest in bronchus supplying the lobe with the lung cancer [21] and is

much smaller in EBC due to the significant dilution of the sample [22,29]. Nevertheless, although concentration of cys-LTs were higher in BALf, Akiyama found significant positive correlation with cys-LTs levels in EBC of patients with idiopathic pulmonary fibrosis [29] whereas Piotrowski demonstrated such positive correlation for 8-isoprostane and LTB4 in patients with sarcoidosis [4]. Similarly, in our study, the concentration of LTB4, cys-LTs and 8-isporostane may reflect their concentration in BALf in patients with diagnosed NSCLC. This finding may depend on the homogeneity of patients' groups in our study and on the higher concentrations of lipids mediators than observed previously in patients with noneoplasmatic diseases. Since different types of cancer secrete different amounts of lipids mediators [21] and extrathoracic malignances as well as metastatic tumor in the lungs from other localizations may affect eicosanoids concentration in BALf [22], we have included exclusively metastatic free patients with planoepithelial cancer.

In this study we did not follow-up our patients with expired biomarkers and we are not aware of the changes of these markers during the course of the disease and treatment. However, Funahashi [22] observed PGE2 decrease in BALf of patients with NSCLC after the successful cancer removal. This topic could be addressed in future studies.

## 6. Conclusion

The present study describes, for the first time, the quantitative assessment of cys-LTs, LTB4, PGE2 and 8-isoprostane measured simultaneously both in EBC and BALF of patients with diagnosed NSCLC. We have demonstrated that in patients with lung cancer with similar smoking habits to healthy smokers, the differences observed in LTB4 and cys-LTs are related mainly to the underlying neoplasmatic disease. As we have found a significant and positive correlation between the levels of LTB4, cys-LTs, and 8-isoprostane in EBC and BALf, we speculate that breath based detection of lipids mediators may help in early detection and monitoring of lung cancer and cancer recurrence.

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## Conflict of interest statement

All authors declare no conflict of interest.

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