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# Inflammatory cytokines and VEGF measured in exhaled breath condensate are correlated with tumor mass in non-small cell lung cancer

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## Abstract

Inflammation mediated by the immune system is known to be important in carcinogenesis and, specifically, T helper 17 cells have been reported to play a role in tumor progression by promoting neo-angiogenesis. The aim of this study was to investigate whether inflammatory cytokines and vascular endothelial growth factor (VEGF) levels in exhaled breath condensate (EBC) and in serum were related to tumor size in patients with non-small cell lung cancer (NSCLC). IL-6, IL-17, TNF- $\alpha$  and VEGF levels were measured in EBC and serum of 15 patients with stage I-IIA NSCLC and in 30 healthy controls by immunoassay. The tumor size was measured by a CT scan. The concentrations of IL-6, IL-17 and VEGF were significantly higher in EBC of patients with lung cancer, compared with controls, while only serum IL-6 concentration was higher in patients compared to controls. A significant correlation ( $r = 0.78, p = 0.001$ ) was observed between EBC levels of IL-6 and IL-17; IL-17 was also correlated to EBC levels of the VEGF ( $r = 0.83, p < 0.001$ ) and TNF- $\alpha$  ( $r = 0.62, p = 0.014$ ). The tumor diameter was significantly correlated with EBC concentrations of VEGF ( $r = 0.58, p = 0.039$ ), IL-6 ( $r = 0.67, p = 0.013$ ) and IL-17 ( $r = 0.66, p = 0.017$ ). Our results show a significant relationship between inflammatory and angiogenic markers, measured in EBC by a non-invasive method, and tumor mass.

## Introduction

Primary lung cancer is the second most frequent tumor in the world and it is the leading cause of cancer-related death [1]. Lung cancer is linked to a long history of smoking and to its accompanying chronic inflammatory response [2]. Different methods for diagnosis and staging are used in clinical practice, based both on imaging (chest x-ray, computed tomography (CT), positron emission tomography (PET)) and blood tests.

Breath analysis is an emerging approach for diagnosing and possibly for monitoring lung cancer, which can be applied directly to exhaled breath or to exhaled breath condensate (EBC). The advantage of breath analysis is that cancer-related biomarkers in exhaled breath may be related to local respiratory tract abnormalities and/or to cancer-related changes in blood chemistry reflected into the breath.

Several studies have used spectrometric methods and/or chemical/nanomaterial sensor arrays [3, 4] to show that the profiles of volatile organic compounds (VOCs) in the breath of lung cancer patients differ from those of healthy persons [5].

It is recognized that both local and systemic inflammation mediated by the immune system play a role in almost every step of carcinogenesis, including tumor initiation and progression [6–8]. Specifically, T helper 17 (Th17) cells have been recently postulated to play a key role in carcinogenesis [9, 10].

Both Th17 cells and the related cytokines (IL-17 and IL-6) have been reported in different tumor tissue sample microenvironment, as well as in blood samples of patients with neoplastic disease [11, 12].

Increased serum IL-17 levels have been related to the spread of malignancies, including prostate, liver and lung cancer [13–15]. An increased number of IL-17-producing cells in neoplastic tissue has been related to poor survival and increased lymphangiogenesis in non-small cell lung cancer (NSCLC) [15].

One of the mechanisms by which IL-17 may be related to cancer diffusion and poor prognosis is its involvement in neo-angiogenesis. Interleukin-17 has been reported to promote the production of pro-angiogenic factors and TNF- $\alpha$  in fibroblast cultures [16]. Actually IL-17 measured in cancer tissue specimens has been reported to be related to the number of blood vessels in ovarian, hepatocellular, gastric and NSCLC [14, 17–19].

Vascular endothelial growth factor (VEGF) is a well-known angiogenic factor, which has been linked to metastasis, poor prognosis and relapse of a wide variety of tumors, including lung cancer [20, 21].

A non-invasive method to assess lung inflammation is to obtain EBC, which collects airway lining fluid, to measure inflammatory markers [22]. To our knowledge, very few studies have measured cytokines in EBC of patients with lung cancer, finding higher concentration of IL-6, VEGF and TNF- $\alpha$  in patients with NSCLC compared to healthy subjects [23–25].

TNF- $\alpha$ , produced by both neoplastic cells and monocyte macrophage cells, has a pro-inflammatory role linked to all steps of tumorigenesis [26], while IL-6 is a pro-inflammatory cytokine which has been implicated in inflammation-associated carcinogenesis [27]. Specifically, IL-6 modulates the expression of genes involved in proliferation, survival and angiogenesis of tumor [28].

The aim of this study is to investigate whether cytokines related to Th-17 cells (IL-6, IL-17), TNF- $\alpha$  and VEGF could be measured in EBC and serum of patients with NSCLC, scheduled for curative lung resection, and whether the EBC levels of these cytokines might be related to the tumor mass.

## **Materials and methods**

### *Patients*

Fifteen consecutive patients, admitted to the Unit of Thoracic Surgery of the University of Torino between January and June 2011 and scheduled for curative lung resection for NSCLC, were enrolled into the study. All the 15 patients were ex-smokers (mean pack-per-year:  $20.2 \pm 2.7$ ). The tumor mass was located peripherally in all the patients. All tumor clinical stages at diagnosis were: IA in eight, IB in four and IIA in three patients [29].

Patients with asthma, COPD, interstitial lung and autoimmune diseases, those who received induction chemotherapy or were on therapy with oral/inhaled corticosteroids were excluded from the study, along with those with a recent (last 8 weeks) or current airway infection.

Thirty healthy subjects matched for age, gender and smoking history were enrolled as control group, provided that they had no cancer history, as well a recent or current airway infection. EBC and blood sample were collected in all the patients and controls.

The study protocol was approved by our Institutional Review Board for human studies (n. 0039 653), and informed consent was obtained from all subjects. The study was conducted in accordance with the Helsinki Declaration.

#### *Collection of exhaled breath condensate (EBC) and blood sample*

EBC collection was performed using the disposable R Tube™ EBC collection system (Respiratory Research Inc., Charlottesville, VA, USA), after thorough rinsing of mouth with water, at an initial condenser temperature of  $-20^{\circ}\text{C}$ . Both patients and controls were investigated in the same lab room. The system consisted of a mouthpiece with a one-way valve and a saliva trap. The subjects, sitting and relaxed, breathed tidally through a disposable mouthpiece, without bacterial filter, for 10 min, wearing a nose clip. They kept their mouth dry during EBC collection by periodically swallowing excess saliva. A nose clip was also applied to exclude possible contamination of the nose. The collection was stopped in the case of coughing or excessive saliva and was restarted when the episode was resolved. For each patient approximately 2 ml of EBC was collected and the samples were immediately stored at  $-80^{\circ}\text{C}$  for later assays [30]. In a subset of healthy controls ( $n = 15$ ) assays had been obtained after 1 and 6 months in order to establish the stability of stored samples (see table 1). The collected EBC samples were stored at  $-80^{\circ}\text{C}$  in polypropylene tubes until analysis. All EBC samples were examined for amylase activity (alpha-Amylase ESP1491300 kit; detection limit  $0.05\text{ mmol l}^{-1}$ ; Boehringer Mannheim, Germany) in order to exclude contamination by saliva. Total protein was measured using a Quantipro BCA assay kit (Sigma Aldrich, Sydney, Australia); lower limit of detection  $4\text{ }\mu\text{g ml}^{-1}$ .

**Table 1.** Mean ( $\text{pg ml}^{-1}$ ) and EBC cytokine levels measured at 1 and 6 months in a subset of healthy controls (n. 15).

	Cytokines	1 month	6 months	p value
EBC	IL-17	1.86 ( $\pm 0.64$ )	1.85 ( $\pm 0.63$ )	NS
	IL-6	0.21 ( $\pm 0.03$ )	0.21 ( $\pm 0.03$ )	NS
	VEGF	35.26 ( $\pm 9.8$ )	34.92 ( $\pm 10.35$ )	NS
	TNF- $\alpha$	0.73 ( $\pm 0.13$ )	0.74 ( $\pm 0.12$ )	NS

A blood sample was obtained in the same day from all patients and controls and serum samples were immediately stored at  $-80^{\circ}\text{C}$  for later assays.

### Cytokines assays

A multiplex immunoassay (Bio-Rad Laboratories Inc., Hercules, CA) was used to analyze serum and EBC cytokines. Cytokines and VEGF were measured using an xMAP technology (Luminex Corp, Austin, TX) on a Bioplex 100 instrument. Data analysis was performed with Bioplex Manager 4.1 software (Bio-Rad Laboratories). An eight-point standard curve in duplicate was included on every 96-well plate. Measurements that were not on the linear part of standard curves (low concentrations) were reported as below the detection limit [31, 32].

Samples from patients and controls were analyzed on the same assay plate, evenly distributed over the plate. The detection limit ( $\text{pg ml}^{-1}$ ) for every variable and the percentage of the samples above the detection limit are reported in table 2. Results with more than 50% of the samples above the detection limit were used for further analysis. All cytokine and growth factor concentrations are expressed in  $\text{pg ml}^{-1}$ .

**Table 2.** Detection limits and measurability of cytokines (expressed as percentage of samples in which cytokines were above the detection limits) in patients and controls.

		Serum		EBC	
	Detection limits ( $\text{pg ml}^{-1}$ )	Positive patients <i>n</i> (%)	Positive controls <i>n</i> (%)	Positive patients <i>n</i> (%)	Positive controls <i>n</i> (%)
IL 6	0.13	13 (87%)	25 (83%)	15 (100%)	30 (100%)
IL 17	0.51	3 (20%)	8 (27%)	15 (100%)	30 (100%)
TNF $\alpha$	0.4	9 (60%)	5 (17%)	15 (100%)	30 (100%)
VEGF	3.79	11 (73%)	28 (93%)	15 (100%)	30 (100%)

### Preoperative evaluation

Pulmonary function tests, arterial blood gas analysis, chest x-rays, chest and upper abdomen CT, bronchoscopy and PET scan represented the normal preoperative workup for patients with lung cancer, according to the more recent clinical guidelines [33].

### CT scan

Multislice thoracic and upper abdomen CT (Lightspeed 16Pro, GE, Milwaukee, WI, USA) was performed in each patient. The nodule size was measured by using an electronic ruler on the workstation and recorded as the average of the maximal length and the width perpendicular to it.

### Statistical analysis

Statistical analysis was performed with the aid of a commercially available statistical package (STATA 10s).  $P < 0.05$  was considered statistically significant. The Kolmogorov–Smirnov test was performed to verify the normal distribution of variables. Histogram plots appeared to have a normal distribution, except for serum IL-17. For the statistical analysis of this we used Mann–Whitney U tests. Two-sided independent-sample *t* tests were used for analysis of the other variables with normal distribution. The correlations

among EBC cytokines and between EBC cytokines and tumor diameter were analyzed by linear regression analysis [34, 35].

## Results

The patients' baseline characteristics are summarized in table 3.

**Table 3.** Comparison between baseline characteristics of patients and controls.

	Patients	Healthy subject	<i>P</i>
Age (range)	63.8 (39–82)	60.1 (41–79)	n.s.
Sex (Female %)	20%	30%	n.s.
FEV1 % predicted (SD)	95.24 (±13.6)	90.37 (±6.9)	n.s.
FVC % predicted (SD)	106.97 (±15.5)	107.65 (±7.1)	n.s.
FEV1/VC×100 (SD)	75.46 (±2.5)	84.13 (±6.7)	n.s.

The mean diameter of tumor was 3.28 (SD ± 2.33) cm; histological examination revealed eight adenocarcinoma, five squamous cell and two undifferentiated carcinoma.

### *EBC cytokines: comparison between patients and controls*

In a subset of healthy controls ( $n = 15$ ), cytokines and VEGF measured in aliquots of the same EBC samples after 1 and 6 months yielded no different results (table 1).

Mean protein concentration of EBC samples was similar in the patients and normal controls ( $13 \pm 2$  and  $14 \pm 1.5$  mcg ml<sup>-1</sup>, respectively –  $p > 0.05$ ).

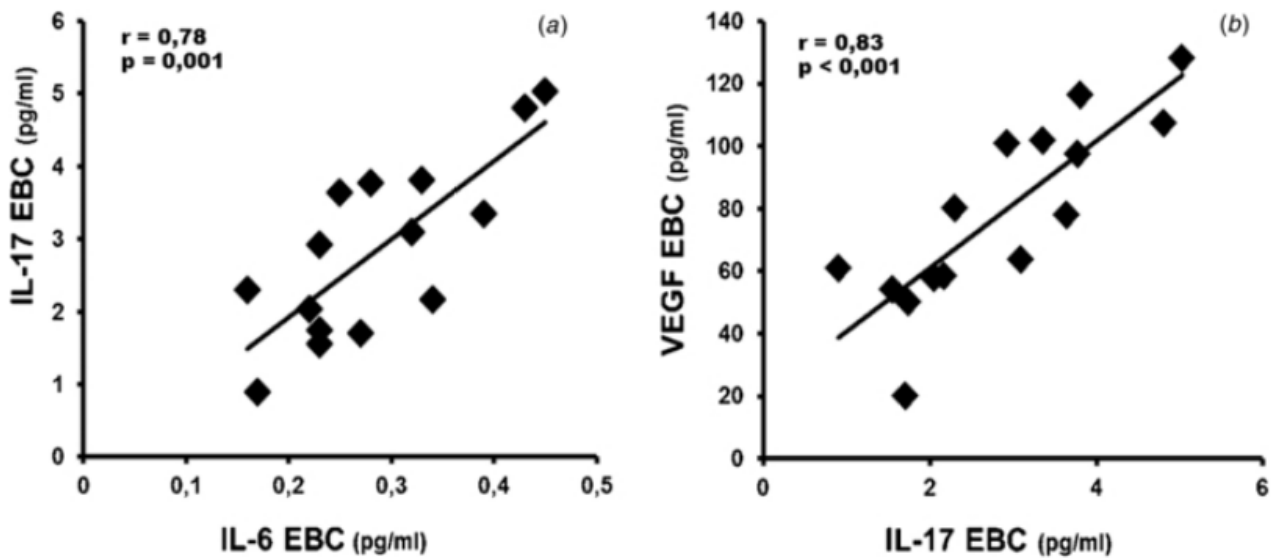
All cytokines and VEGF were measurable in more than 50% of EBC samples of patients and controls, while only IL-6 and VEGF were measurable above the detection limit in the serum samples of both patients and controls (see table 2). The concentration of IL-6, IL-17 and VEGF were significantly higher in EBC of patients compared with controls, while only IL-6 serum concentration was higher in patients compared to controls (see table 4). No relationship was observed between serum and EBC concentration of cytokines, both in patients and healthy controls.

**Table 4.** Mean ( $\text{pg ml}^{-1}$ ) and SD serum and EBC cytokine levels in patients compared to controls.

		Patients	Controls	<i>p</i> value
Serum	IL-6	1.21 ( $\pm 0.99$ )	0.15 ( $\pm 0.06$ )	<0.016
	VEGF	26.28 ( $\pm 19.29$ )	24.77 ( $\pm 24.00$ )	NS
	TNF- $\alpha$	0.67 ( $\pm 0.48$ )	non detectable	N/A
EBC	IL-17	2.85 ( $\pm 1.22$ )	1.83 ( $\pm 0.57$ )	<0.001
	IL-6	0.29 ( $\pm 0.08$ )	0.21 ( $\pm 0.04$ )	<0.001
	VEGF	78.45 ( $\pm 29.45$ )	49.26 ( $\pm 26.55$ )	0.002
	TNF- $\alpha$	0.75 ( $\pm 0.29$ )	0.76 ( $\pm 0.13$ )	NS

*Inter-relationships of EBC cytokines in patients*

A significant correlation ( $r = 0.78$ ,  $p = 0.001$ ) was observed between EBC levels of IL-6 and IL-17 (figure 1(a)), which was also correlated to EBC levels of VEGF ( $r = 0.83$ ,  $p < 0.001$ ) (figure 1(b)).



**Figure 1.** Correlation between EBC levels of IL-6 and IL-17 (a), and correlation between EBC level of IL-17 and VEGF (b).

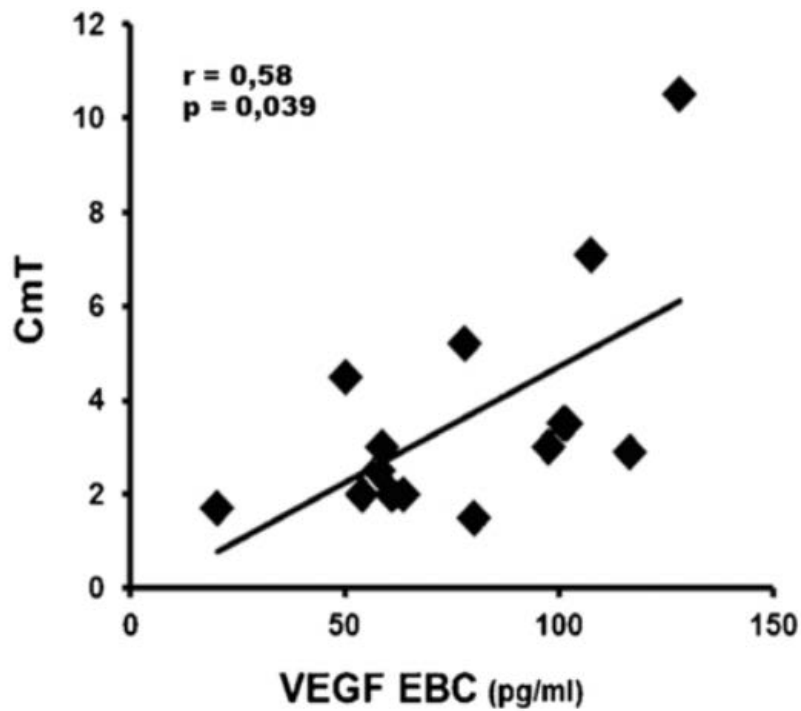
Levels of EBC IL-17 of patients were significantly correlated to TNF- $\alpha$  levels ( $r = 0.62$ ,  $p = 0.014$ ). TNF- $\alpha$  was also significantly correlated with VEGF in EBC ( $r = 0.62$ ,  $p = 0.013$ ). No correlations were observed among



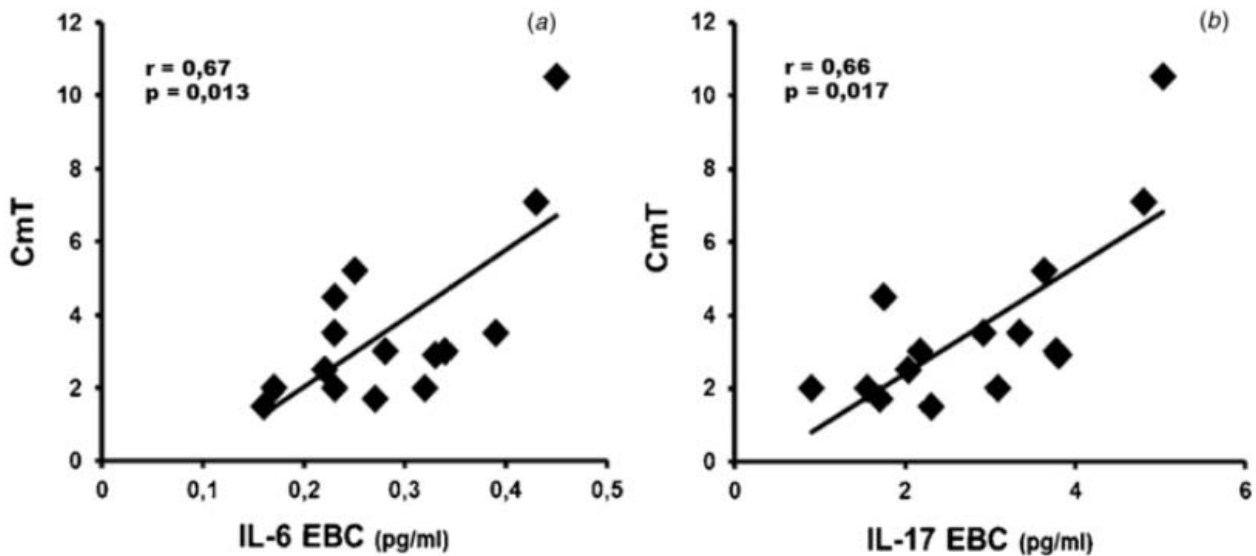
serum cytokines concentrations both in patients and controls and no correlations were observed among EBC cytokines in controls.

*Relationship between tumor diameter and cytokines*

The tumor diameter was significantly correlated with EBC concentrations of VEGF ( $r = 0.58, p = 0.039$ ) (figure 2), IL-6 ( $r = 0.67, p = 0.013$ ) (figure 3(a)) and IL-17 ( $r = 0.66, p = 0.017$ ) (figure 3(b)). No correlation was found between the tumor diameter and serum cytokine.



**Figure 2.** Correlation between tumor diameter at CT scan and EBC concentrations of VEGF.



**Figure 3.** Correlation between tumor diameter at CT scan and EBC concentrations of IL6 (a) and IL-17 (b).

## Discussion

All the biomarkers were found in concentrations above the detection limit in all the EBC samples of both patients and controls, while in blood samples IL-17 could be measured only in 20% of samples. As EBC was collected without fractioning exhaled air [36], contamination from dead space could not be excluded.

Higher levels of IL-17, IL-6 and VEGF were measured in EBC of patients with NSCLC compared to controls, and a significant positive correlation was observed between the tumor diameter, detected by lung CT-scan, and EBC levels of the above reported cytokines. Higher concentration of VEGF and IL-6 in EBC of patients with NSCLC compared to controls had been previously reported by Gessner *et al* [37] and Carpagnano *et al* [23], respectively. While VEGF levels we measured are comparable to those reported by Gessner, IL-6 levels reported by Carpagnano are somewhat higher than ours, possibly due to the different technique (specific enzyme immunoassay kit) used by Carpagnano. Conversely, to our knowledge, this is the first demonstration of IL-17 detection in EBC of patients with NSCLC.

Increased levels of EBC IL-6 and IL-17 have also been observed in patients with COPD and asthma respectively [38–40]; that is the reason why chronic airway disease was an exclusion criterion for our patients.

Our finding is particularly interesting, as IL-17 has been reported to promote the production of pro-angiogenic factors in fibroblast cultures [16] and IL-17, measured in cancer tissue specimens, has been related to the number of blood vessels in a variety of tumors, including NSCLC) [14, 17–19]. Actually, the correlation we observed between IL-17 and VEGF and TNF- $\alpha$  in EBC of patients, is in agreement with the reported effect of IL-17 on angiogenesis.

Of particular interest also is the correlation observed between IL-6 and IL-17 in patients' EBC, but neither in serum nor in control subjects

Luckheeram [41], in a recent review on mechanisms of differentiation of T helper lymphocytes, describes the role of IL-6 in the production of Th17. Interleukin 6, in synergism with TGF- $\beta$  and IL-23, could stimulate the development and expansion of Th17. The consequent production of IL-17, in turn, would further increase IL-6 production, thus creating a self-maintenance cycle [42].

The correlation between cytokines, observed in EBC of patients, points to an inflammatory network promoted locally by the tumor. Unfortunately, we could not assess EBC levels of IL-23 and TGF- $\beta$ , as their concentration was below the detection limit only in a few EBC samples (data not shown).

We did not observe any correlation between serum and EBC levels of cytokines, suggesting that inflammatory changes are mainly limited to the target organ (lung) without systemic involvement, at least in the early stages of disease. On the other hand, increased IL-6 and IL-8 serum concentration had been reported in the greatest proportion of stage I NSCLC patients by Kaminska *et al* [43]. The high capacity of breath analysis to detect early lung cancer was demonstrated by Peled *et al* [44] who investigated the profiles of VOCs, determined by gas chromatography/mass spectrometry (GC-MS) combined with solid phase microextraction (SPME) and by a chemical nanoarray in breath samples taken from 72 patients with pulmonary nodules in a prospective trial.

Actually serum IL-6 levels, a marker of non-specific inflammatory response, have been found higher in patients than in controls. In previous studies, increased levels of circulating proinflammatory cytokines, IL-6 and IL-8, were reported to be associated with lung cancer [45, 46].

The positive relationships between the tumor diameter and EBC levels of IL-6, IL-17 and VEGF suggest that the same cytokines are produced either by the tumor itself or by a more extensive tissue infiltration of inflammatory cells.

This relationships between the Th17-cytokines EBC level and tumor diameter may support the previous observation of Carpagnano on the presence of a relationship between the level of cytokines in EBC and stage of disease [23]. The design of our study was planned to select only patients with early-stage disease, so that further studies are needed to verify the relationship between IL-17 and the disease stage.

Our results, for the first time, underline the strict relationship between inflammatory and angiogenic markers, measured non-invasively in EBC, and tumor mass. Theoretically, these cytokines could be produced either by the tumor itself or by a more extensive tissue infiltration of inflammatory cells.

## **Conclusion**

Our data demonstrate that, in the early stages of NSCLC, inflammatory changes are detectable in the respiratory system, and that cytokine levels, measured in EBC, but not in serum, correlate with the tumor mass.

Further studies are needed to evaluate the changes in EBC inflammatory markers following surgical resection and the clinical usefulness of breath testing in the follow-up of patients with lung cancer.

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The authors have declared no conflicts of interest.

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