

## **Exhaled and non-exhaled non-invasive markers for assessment of respiratory inflammation in patients with stable COPD and healthy smokers**

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## **Abstract**

We aimed at comparing exhaled and non-exhaled non-invasive markers of respiratory inflammation in patients with COPD and healthy subjects and define their relationships with smoking habit. Forty-eight patients with stable COPD who were ex-smokers, 17 patients with stable COPD who were current smokers, 12 healthy current smokers and 12 healthy ex-smokers were included in a cross-sectional, observational study. Biochemical [prostaglandin (PG) E<sub>2</sub>, 15-F<sub>2t</sub>-isoprostane, fraction of exhaled nitric oxide (F<sub>E</sub>NO)] and cellular (sputum cell counts) inflammatory outcomes and functional (spirometry) outcomes were measured in various biological matrices including exhaled breath condensate (EBC), exhaled breath, sputum supernatants, and urine. Sputum PGE<sub>2</sub> was elevated in both groups of smokers compared with ex-smoker counterpart (COPD:  $P < 0.02$ ; healthy subjects:  $P < 0.03$ ), whereas EBC PGE<sub>2</sub> was elevated in current ( $P = 0.0065$ ) and ex-smokers with COPD ( $P = 0.0029$ ) versus healthy ex-smokers. EBC 15-F<sub>2t</sub>-isoprostane, a marker of oxidative stress, was increased in current and ex-smokers with COPD ( $P < 0.0001$  for both) compared with healthy ex-smokers, whereas urinary 15-F<sub>2t</sub>-isoprostane was elevated in both smoker groups (COPD:  $P < 0.01$ ; healthy subjects:  $P < 0.02$ ) versus healthy ex-smokers. F<sub>E</sub>NO was elevated in ex-smokers with COPD versus smoker groups ( $P = 0.0001$  for both). These data suggest that the biological meaning of these inflammatory markers depends on type of marker and biological matrix in which is measured. An approach combining different types of outcomes can be used for assessing respiratory inflammation in patients with COPD. Large studies are required to establish the clinical utility of this strategy.

Keywords: exhaled breath condensate, sputum, prostaglandin E<sub>2</sub>, 15-F<sub>2t</sub>-isoprostane, fraction of exhaled nitric oxide, respiratory inflammation, chronic obstructive pulmonary disease

Abstract word count: 252

## Introduction

The pathophysiological role of prostaglandin (PG) E<sub>2</sub>, an endogenous eicosanoid which is generally considered pro-inflammatory [1,2], but can have anti-inflammatory effects in the respiratory system [3,4], and 15-F<sub>2t</sub>-isoprostane, a marker of lipid peroxidation [5], in chronic obstructive pulmonary disease (COPD) is largely not defined. PGE<sub>2</sub> and 15-F<sub>2t</sub>-isoprostane are detected in the exhaled breath condensate (EBC) in healthy subjects [6,7] and their concentrations are elevated in this biological fluid in patients with stable COPD [8-10]. Compared with healthy chronic smokers and healthy nonsmokers, sputum PGE<sub>2</sub> has been reported elevated in current smokers with COPD, but not in COPD patients who were ex-smokers [11]. Sputum 15-F<sub>2t</sub>-isoprostane concentrations were found elevated in patients with stable COPD as compared with healthy smokers, never-smokers and ex-smokers [12].

However, the relationships between PGE<sub>2</sub> and 15-F<sub>2t</sub>-isoprostane concentrations in EBC and sputum supernatants, which reflect inflammation at different levels of the respiratory system [13,14], is not defined. Elevated urinary 15-F<sub>2t</sub>-isoprostane concentrations in patients with COPD have been reported [15], but their relationships with 15-F<sub>2t</sub>-isoprostane concentrations in EBC and sputum are unknown.

In the present study, the primary objective was to compare exhaled and non-exhaled non-invasive markers of respiratory inflammation in patients with COPD and healthy subjects and define their relationships with smoking habit. Secondary objectives were to clarify the relationships between PGE<sub>2</sub> concentrations in EBC and sputum supernatants, between 15-F<sub>2t</sub>-isoprostane concentrations in EBC, sputum supernatants and urine, and between lung and systemic inflammation by measuring independent markers of airway (fraction of exhaled nitric oxide (F<sub>E</sub>NO), sputum inflammatory cells) and systemic inflammation/oxidative stress (urinary 15-F<sub>2t</sub>-isoprostane).

## **Methods**

### ***Study subjects***

Forty-eight patients with stable COPD who were ex-smokers, 17 patients with stable COPD who were current smokers, 12 healthy current smokers and 12 healthy ex-smokers were studied. Diagnosis and classification of COPD was based on GOLD guidelines [16]. Diagnosis of COPD was based on history of smoking, symptoms of dyspnea, cough and sputum production, and the presence of post-bronchodilator forced expiratory volume in one second ( $FEV_1$ )/forced vital capacity (FVC) < 70% [16]. Patients had mild to severe COPD (GOLD stage 1-3) based on severity of airflow limitation [16]. Healthy subjects and patients with COPD who were ex-smokers and had been stopped smoking for at least 6 months. Healthy current smokers and patients with COPD who were current smokers were not allowed to smoke in the previous 12 h before study procedures. All patients with COPD had no history of asthma or other respiratory diseases, negative reversibility test to bronchodilators (inhalation of salbutamol at a dose of 400 µg), and negative skin prick tests results to common aeroallergens. Study subjects had stable COPD with no exacerbations or upper respiratory infections in the previous 4 weeks. Details of pharmacological treatment are shown in Table 1. Patients with COPD were considered not on ICS if they were being steroid-naïve or had been stopping ICS for at least 8 weeks. All patients with COPD had never been on oral corticosteroids or had been stopping oral corticosteroids for at least 8 weeks.

**Table 1.** Subject characteristics.

	Healthy ex-smokers	Healthy smokers	COPD ex-smokers	COPD current smokers
N	12	12	48	17
Gender, male/female	9/3	10/2	36/12	12/5
Age	56 ± 3.5	48 ± 3.3 <sup>c</sup>	69 ± 1.3 <sup>a</sup>	60 ± 2.5 <sup>b,d</sup>
FEV <sub>1</sub> , L	3.4 ± 0.2	3.7 ± 0.2	1.6 ± 0.1 <sup>a</sup>	1.8 ± 0.1 <sup>a</sup>
FEV <sub>1</sub> , % pred	102.8 ± 3.0	100.9 ± 2.6	58.0 ± 2.4 <sup>a,e</sup>	66.5 ± 3.5 <sup>a</sup>
FVC, L	4.1 ± 0.3	4.7 ± 0.2	2.6 ± 0.1 <sup>a</sup>	2.8 ± 0.2 <sup>a</sup>
FVC, % pred	103.0 ± 3.4	106.0 ± 1.8	76.2 ± 2.3 <sup>a</sup>	80.4 ± 3.8 <sup>a</sup>
FEV <sub>1</sub> /FVC	80.9 ± 1.4	77.9 ± 1.4	59.7 ± 1.5 <sup>a,e</sup>	65.2 ± 1.8 <sup>a</sup>
Pack-years	18.0 (9.8-21.5)	22.5 (11.3-41.3)	40.0 (23.4-60.0) <sup>c</sup>	26.0 (20.0-57.5)
GOLD stage (1/2/3)	-	-	35/12/1	15/2/0
ICS, yes/no	-	-	12/36	7/10
LABA, yes/no	-	-	15/33	7/10
LAMA, yes/no	-	-	6/42	2/15

<sup>a</sup>Data are expressed as mean ± SEM, except pack-years which are expressed as median and 25%-75% interquartile range. <sup>a</sup>, p<0.001, compared with healthy smokers and healthy ex-smokers;

<sup>b</sup>, P<0.001 compared with healthy smokers; <sup>c</sup>, P<0.01, compared with healthy ex-smokers;

<sup>d</sup>, P<0.01, compared with COPD ex-smokers; <sup>e</sup>, P<0.05, compared with COPD current smokers

Patients with COPD who were on ICS received fluticasone or the equivalent of fluticasone at a dose of 500 µg twice a day. Patients with COPD who were on LABA received the salmeterol or the equivalent of salmeterol at a dose of 50 µg twice a day. Patients with COPD who were on LAMA received tiotropium bromide at a dose of 18 µg once day via a DPI. Abbreviations: DPI, dry powder inhaler; FEV<sub>1</sub>, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroids; LABA, long-acting beta<sub>2</sub>-receptor agonists; LAMA, long-acting muscarinic receptor antagonists.

### ***Study design***

This was a national, multicentre, observational, cross-sectional study. Participants were recruited in three centres: University of Modena and Reggio Emilia, COPD outpatient clinic, Modena, Italy; University of Parma, COPD outpatient clinic, Parma, Italy; Clinical Pharmacology and COPD outpatient clinic, University Hospital Agostino Gemelli, Catholic University of the Sacred Heart, Rome, Italy. Subjects attended on occasion for clinical examination, urinary collection, F<sub>E</sub>NO measurement, EBC collection, pulmonary function tests, sputum induction and skin prick testing which were performed in this order.

Informed consent was obtained from patients. The study was approved by the Ethics Committee of the Catholic University of the Sacred Heart, Rome, Italy, of University of Modena and Reggio Emilia, Italy, and of University of Parma, Italy.

### ***Exhaled breath condensate sampling***

EBC was collected using a condenser (Ecoscreen, Jaeger, Hoechberg, Germany) as previously described [17] and according to standardised procedures [13]. Exhaled breath entered and left the chamber through one-way valves at the inlet and outlet, thus keeping the chamber closed. Subjects breathed tidally through a mouthpiece connected to the condenser for 15 minutes. EBC samples were stored at  $-80^{\circ}\text{C}$  until analytical measurement. The condenser has a saliva trap to reduce the chance of salivary contamination.  $\alpha$ -Amylase concentrations were measured in all EBC samples by an in vitro colorimetric method to check for possible salivary contamination (Roche Diagnostics, Basel, Switzerland).

### ***F<sub>E</sub>NO measurement***

F<sub>E</sub>NO was measured with the NIOX system (Aerocrine, Stockholm, Sweden) with a single breath on-line method at constant flow of 50 ml/sec according to American Thoracic Society guidelines [19,20]. Exhalations were repeated after 1-minute relaxation period until the performance of three F<sub>E</sub>NO values varies less than 10%. F<sub>E</sub>NO measurements were obtained before spirometry.

### ***Measurement of PGE<sub>2</sub> and 15-F<sub>2t</sub>-isoprostane in sputum supernatants and EBC***

15-F<sub>2t</sub>-isoprostane and PGE<sub>2</sub> concentrations were measured by radioimmunoassays (RIAs) developed in our laboratory which were previously validated and compared with GC/MS and high performance liquid chromatography (HPLC) [21,22]. These RIAs have a sensitivity of 2 pg/ml. Intra-assay and

inter-assay coefficient of variation for 15-F<sub>2t</sub>-isoprostane were < 2% and < 3%, respectively; intra-assay and inter-assay coefficient of variation for PGE<sub>2</sub> were < 4% and < 5%, respectively [21].

### ***Pulmonary function***

Spirometry was performed with a Pony FX spirometer (Cosmed, Rome, Italy) and the best of at least three acceptable FVC manoeuvres chosen [23]. Acceptable repeatability is achieved when both the two largest FVC and FEV<sub>1</sub> values, respectively, are within 0.15 L of each other (0.1 L for subjects with a FVC ≤ 1.0 L) [23].

### ***Sputum induction, processing and analysis***

Sputum induction, processing and analysis were performed according the ERS guidelines [24-25]. Baseline FEV<sub>1</sub> was recorded before sputum induction. Subjects were pre-treated with inhaled salbutamol (400 µg), and, after 10 min, a spirometry was repeated [25]. Subjects were asked to inhale hypertonic saline (3%) for 5 min and, then, to rinse their mouths and try to expectorate into a sterilized box [25]. Five-minute inhalation sessions were repeated 4 times for a total of 20 minutes [25]. A spirometry was performed after each inhalation session to detect significant fall of FEV<sub>1</sub>. The procedure was stopped when approximately 1 g of plugs was collected, or if patients had symptoms or/and if FEV<sub>1</sub> was reduced more than 20% over baseline values [25]. Sputum was processed within 2 hours to ensure optimum cell counting and staining, with the sample always kept in ice [26].

100-500 mg sputum free of salivary contamination was selected for sputum analysis. Dithiothreitol (DTT) 0.1% was added to sputum samples which were kept in a shaking rocker at room temperature for 20 minutes for sample homogenization [26]. Samples were filtered through a 48 µm nylon mesh into a pre-weighed conical tube and filtrate was weighed. Total cell count was performed manually

using a haemocytometer and cell viability was assessed by the trypan blue exclusion method before centrifugation [26].

To separate cell pellet from sputum supernatants, samples were centrifuged at 4°C for 10 minutes with a centrifugal force of 1200 x g [26]. Sputum supernatant samples were collected and stored at -80°C for measurement of PGE<sub>2</sub> and 15-F<sub>2t</sub>-isoprostane concentrations. Cell pellets were resuspended in PBS buffer and cell concentrations were adjusted to 1 x 10<sup>6</sup> cells/ml. Cytospins were prepared by adding 40-60 µl of cell resuspension to each cytospin and using a Shandon cytocentrifuge at 22 x g for 6 minutes [26]. Cytospins were stained for differential cell counts using Giemsa staining [26]. The differential cell counts was performed by counting a minimum of 400 nonsquamous cells and reported as the relative numbers of eosinophils, neutrophils, macrophages, lymphocytes, and bronchial epithelial cells, expressed as a percentage of total nonsquamous cells [26]. The percentage of squamous cells was reported separately. Slides with squamous cells > 30% of total cells were discarded. Slides were read blindly by two qualified and fully trained physicians. Monthly quality control was performed including internal slide reading and equipment calibration.

#### ***Measurement of urinary 15-F<sub>2t</sub>-isoprostane***

Morning urinary samples (30-50 ml) were collected, additioned with 1 mM of the antioxidant compound 4-hydroxy TEMPO (Sigma Chem., Milan, Italy), snap frozen and kept at -80°C until analyzed. 2 ml of the sample were acidified with acetic acid and additioned with 20.000 dpm of internal standard (<sup>3</sup>H)-15-F<sub>2t</sub>-isoprostane. Following centrifugation, 15-F<sub>2t</sub>-isoprostane was purified by a double extraction procedure [22] with modifications as previously described [27], followed by quantification by an enzyme immunoassay (EIA) [22].

The first extraction was performed using bond-elut C<sub>18</sub> cartridges activated with 1 ml methanol and 1 ml water. After sample loading, the column was washed with 1 ml each of water, hexane and a 75:25



solution of hexane:ethylacetate. Samples were then eluted with 1 ml ethylacetate [22,27]. The second extraction was performed using bond-elut Si cartridges activated with 1 ml each of ethylacetate and 1:1 hexane:ethylacetate [22,27]. Samples were additionally with 1 ml hexane, loaded and eluted with 1 ml methanol:ethylacetate, 20:80 [22,27]. The eluate was frozen at -20°C overnight and taken to dryness using a refrigerated centrifuge Savant, mod. SpeedVac. The dry residue was redissolved in 1.8 ml of EIA buffer and sonicated to allow optimal solubilisation [22,27]. An aliquot (0.2 ml) was used for recovery evaluation and different aliquots of the remaining sample were used for EIA using commercially available reagents (Cayman Chem. Co. Ann Arbor, MI, USA; SPI-BIO, Saclay, France). Results were corrected for the recovery of radiolabeled tracer and expressed as pg/mg creatinine.

### ***Skin testing***

Atopy was assessed by skin prick tests for common aeroallergens (Stallergenes, Antony, France).

### ***Statistical analysis***

Normally distributed values were expressed as mean  $\pm$  SEM. Non-parametric values were expressed as medians and interquartile ranges (25<sup>th</sup> and 75<sup>th</sup> percentiles). Between-group comparison was performed by one-way ANOVA or Kruskal-Wallis test based on normal or non-parametric data distribution, respectively [28]. Correlation was expressed as Spearman's correlation coefficient. P value < 0.05 was considered statistically significant. Data were analyzed using Prism 5.0 (GraphPad Software, Inc., USA).

## **Results**

Subject characteristics are shown in Table 1. Both groups of patients with COPD were older than healthy control subjects ( $P < 0.001$ ) (Table 1). Healthy smokers were younger than healthy ex-smokers ( $P < 0.01$ ) (Table 1).

$\alpha$ -Amylase concentrations were undetectable in all EBC samples, thus excluding significant salivary contamination.

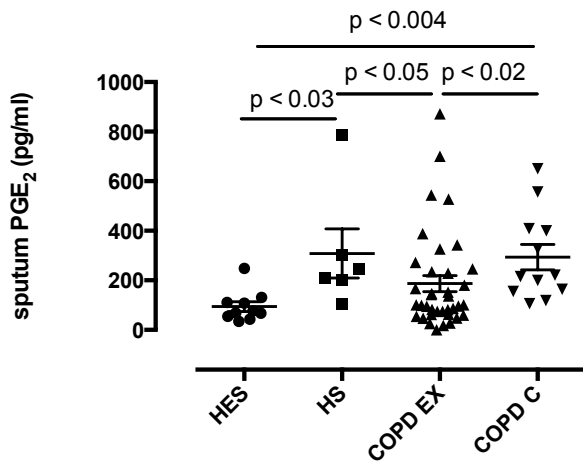
### ***Pulmonary function***

Compared with healthy ex-smokers and healthy smokers, absolute pre-bronchodilator FEV<sub>1</sub> values were reduced in patients with COPD who were ex-smokers ( $P < 0.001$ ) and current smokers ( $P < 0.001$ ). Pre-bronchodilator FEV<sub>1</sub>% of the predicted value was lower in patients with COPD who were ex-smokers and current smokers compared with healthy ex-smokers ( $P < 0.001$ ) and healthy smokers ( $P < 0.001$ ). In our study, patients with COPD who were ex-smokers had lower FEV<sub>1</sub>% of the predicted value than patients with COPD who were current smokers ( $P < 0.05$ ) (Table 1). Absolute FVC, FVC% of the predicted value, and FEV<sub>1</sub>/FVC ratio were reduced in both groups of COPD patients compared with healthy control subjects ( $P < 0.001$ ), but were similar in COPD groups (Table 1).

### ***PGE<sub>2</sub> concentrations in sputum supernatants***

PGE<sub>2</sub> concentrations in sputum supernatants were measured in 37 patients with COPD who were ex-smokers, 12 patients with COPD who were current smokers, 6 healthy smokers and 10 healthy ex-smokers. The lower number of sputum samples analysed compared with the number of recruited patients reflects failure to induce sputum. PGE<sub>2</sub> concentrations, expressed as median values and interquartile range, were measurable in all sputum supernatant samples.

PGE<sub>2</sub> concentrations in sputum supernatants were as follows: 71.7 (46.2-125.6) pg/ml in healthy ex-smokers, 227.5 (178.4-422.6) pg/ml in healthy smokers, 100.8 (62.9-240.4) pg/ml in patients with COPD who were ex-smokers and 218.2 (157.3-406.6) pg/ml in patients with COPD who were current smokers (Figure 1).



**Figure 1.** PGE<sub>2</sub> concentrations in sputum supernatants in healthy ex-smokers (HES) (n = 10), healthy smokers (HS) (n = 6), patients with COPD who were ex-smokers (COPD EX) (n = 37), and patients with COPD who were current smokers (COPD C) (n = 12).

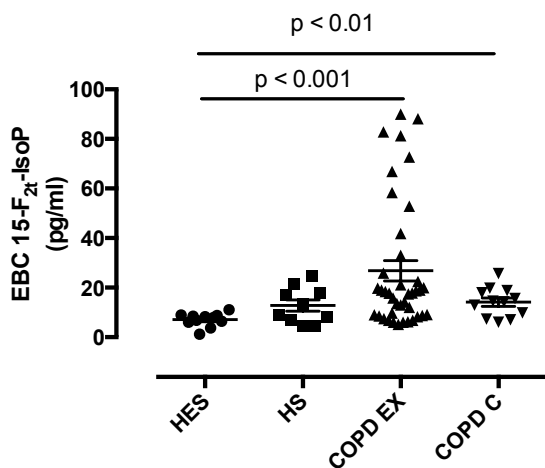
Compared with ex-smokers with COPD and healthy ex-smokers, PGE<sub>2</sub> concentrations in sputum supernatants were elevated in current smokers with COPD (P < 0.02 and P < 0.004, respectively) and in healthy current smokers (P < 0.05 and P < 0.03, respectively) (Figure 1). Similar sputum PGE<sub>2</sub> concentrations were observed in patients with COPD who were current smokers and healthy current smokers (P = 0.96).

### ***15-F<sub>2t</sub>-isoprostane concentrations in exhaled breath condensate***

15-F<sub>2t</sub>-isoprostane concentrations in EBC were measured in 40 ex-smokers with COPD, 13 current smokers with COPD, 10 healthy smokers and 12 healthy ex-smokers. EBC 15-F<sub>2t</sub>-isoprostane

concentrations were measurable in all samples, except two samples which were obtained from a healthy ex-smoker subject and from a current smoker with COPD.

15-F<sub>2t</sub>-isoprostane concentrations, expressed as median values and interquartile range, were as follows: 8 (6.0-8.8) pg/ml in healthy ex-smokers, 11.2 (6.4-18.8) pg/ml in healthy current smokers, 17.8 (8.8-31.2) pg/ml in patients with COPD who were ex-smokers and 14.5 (7.9-18.6) pg in patients with COPD who were current smokers (Figure 2). Both group of patients with COPD had EBC 15-F<sub>2t</sub>-isoprostane concentrations higher than those observed in healthy ex-smokers (ex-smokers with COPD: P < 0.001; current smokers with COPD: P < 0.01), but similar to those observed in healthy current smokers (P = 0.11) (Figure 2).



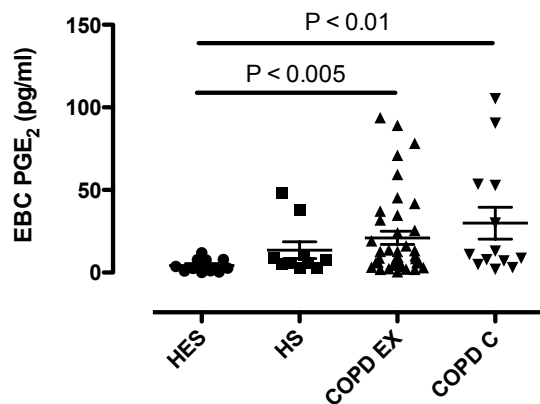
**Figure 2.** 15-F<sub>2t</sub>-isoprostane concentrations in EBC in healthy ex-smokers (HES) (n = 11), healthy smokers (HS) (n = 10), patients with COPD who were ex-smokers (COPD EX) (n = 40), and patients with COPD who were current smokers (COPD C) (n = 12).

There was a trend towards increased EBC 15-F<sub>2t</sub>-isoprostane concentrations in healthy current smokers compared with healthy ex-smokers which did not reach statistical significance (P = 0.098). Similar EBC 15-F<sub>2t</sub>-isoprostane concentrations were observed in both groups of patients with COPD (P = 0.30) (Figure 2).

### *PGE<sub>2</sub> concentrations in exhaled breath condensate*

PGE<sub>2</sub> concentrations in EBC, expressed as median values and interquartile range, have been measured in 40 patients with COPD who were ex-smokers, 13 patients with COPD who were current smokers, 10 healthy current smokers and 11 healthy ex-smokers. PGE<sub>2</sub> concentrations were detected in all EBC samples.

EBC PGE<sub>2</sub> concentrations were as follows: 2.7 (1-7.7) pg/ml in healthy ex-smokers, 6.7 (4.9-17.6) pg/ml in healthy current smokers, 9.3 (6.0-30.3) pg/ml in patients with COPD who were ex-smokers and 10.9 (5.9-53.1) pg/ml in patients with COPD who were current smokers (Figure 3).



**Figure 3.** PGE<sub>2</sub> concentrations in EBC in healthy ex-smokers (HES) (n = 11), healthy smokers (HS) (n = 10), patients with COPD who were ex-smokers (COPD EX) (n = 40), and patients with COPD who were current smokers (COPD C) (n = 13).

EBC PGE<sub>2</sub> concentrations were elevated in both groups of patients with COPD (current smokers: P = 0.0065; ex-smokers: 0.0029) compared with healthy ex-smokers, but not healthy current smokers (current smokers with COPD: P = 0.40; ex-smokers with COPD: P = 0.21). Similar EBC PGE<sub>2</sub> concentrations were observed in current smokers and ex-smokers with COPD (P = 0.53). PGE<sub>2</sub> increase in healthy current smokers compared with healthy ex-smokers did not reach statistical significance (P = 0.067).

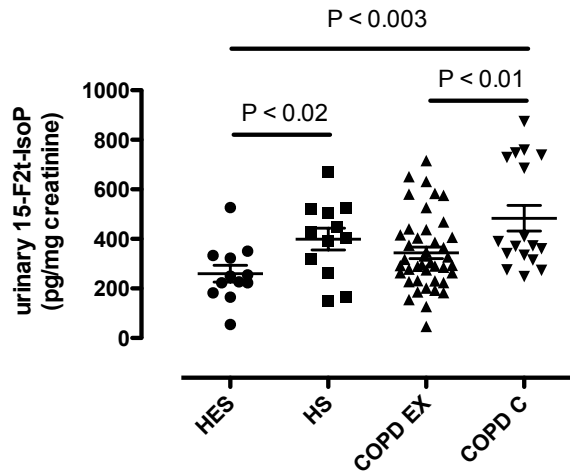
### ***15-F<sub>2t</sub>-isoprostane concentrations in sputum supernatants***

15-F<sub>2t</sub>-isoprostane concentrations were measured in sputum supernatants in 36 patients with COPD who were ex-smokers, 12 patients with COPD who were current smokers, 6 healthy current smokers and 9 healthy ex-smokers. 15-F<sub>2t</sub>-isoprostane was undetectable in 2 healthy ex-smokers and 6 patients with COPD who were ex-smokers. 15-F<sub>2t</sub>-isoprostane concentrations, expressed as median values and interquartile range, were as follows: 2.0 (1.3-3.2) pg/ml in healthy ex-smokers, 6.0 (5.7-18.3) pg/ml in healthy current smokers, 6.0 (2.8-20.3) pg/ml in patients with COPD who were ex-smokers and 11.0 (6.0-14.6) pg/ml in patients with COPD who were current smokers. Patients with COPD who were current smokers had higher sputum 15-F<sub>2t</sub>-isoprostane concentrations than healthy ex-smokers ( $P = 0.0007$ ), but similar to those observed in healthy smokers ( $P = 0.34$ ) and patients with COPD who were ex-smokers ( $P = 0.11$ ).

### ***Urinary 15-F<sub>2t</sub>-isoprostane***

Urinary 15-F<sub>2t</sub>-isoprostane concentrations were measured in 42 patients with COPD who were ex-smokers, 17 patients with COPD who were current smokers, 12 healthy current smokers and 12 healthy ex-smokers. 15-F<sub>2t</sub>-isoprostane was detected in all urinary samples. Urinary 15-F<sub>2t</sub>-isoprostane concentrations were as follows:  $259.4 \pm 33.7$  pg/mg creatinine, mean  $\pm$  SEM, in healthy ex-smokers,  $399.4 \pm 44.3$  pg/mg creatinine in healthy smokers,  $343.6 \pm 23.2$  pg/mg creatinine in patients with COPD who were ex-smokers, and  $483.4 \pm 51.9$  pg/mg creatinine in patients with COPD who were current smokers (Figure 4). Compared with healthy ex-smokers, urinary 15-F<sub>2t</sub>-isoprostane was elevated in healthy current smokers ( $P < 0.02$ ) (Figure 4). Current smokers with COPD had higher urinary 15-F<sub>2t</sub>-isoprostane concentrations than ex-smokers with COPD ( $P < 0.01$ ) and healthy ex-smokers ( $P < 0.003$ ), but similar to those observed in healthy current smokers (Figure 4). There was no

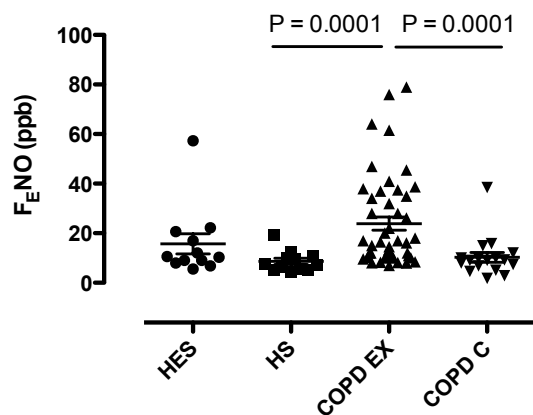
difference in urinary 15-F<sub>2t</sub>-isoprostane concentrations between current smokers with COPD and healthy current smokers (P = 0.26) (Figure 4).



**Figure 4.** Urinary 15-F<sub>2t</sub>-isoprostane concentrations in EBC in healthy ex-smokers (HES) (n = 12), healthy smokers (HS) (n = 12), patients with COPD who were ex-smokers (COPD EX) (n = 42), and patients with COPD who were current smokers (COPD C) (n = 17).

### *F<sub>E</sub>NO*

F<sub>E</sub>NO was measured in 47 patients with COPD who were ex-smokers, 17 patients with COPD who were current smokers, 12 healthy current smokers and 12 healthy ex-smokers. F<sub>E</sub>NO concentrations were detected in all study subjects (Figure 5). F<sub>E</sub>NO concentrations were elevated in patients with COPD who were ex-smokers [16.1 (10.8-35.0) ppb, median and interquartile range] compared with patients with COPD who were current smokers [9.0 (5.9-11.0) ppb, P = 0.0001] and healthy smokers [7.4 (5.1-10.8) ppb, P = 0.0001] (Figure 5). Compared with healthy ex-smokers [10.4 (8.3-19.7) ppb], ex-smokers with COPD [16.1 (10.8-35.0) ppb] had higher F<sub>E</sub>NO values, a difference which was very close to statistical significance (P = 0.0546).



**Figure 5.** Fraction of exhaled nitric oxide ( $F_{E}NO$ ) concentrations in healthy ex-smokers (HES) ( $n = 12$ ), healthy smokers (HS) ( $n = 12$ ), patients with COPD who were ex-smokers (COPD EX) ( $n = 47$ ), and patients with COPD who were current smokers (COPD C) ( $n = 17$ ).

### *Cellular analysis of induced sputum*

Cellular analysis of induced sputum was performed in 9 healthy ex-smokers, 6 healthy smokers, 29 patients with COPD who were ex-smokers, and 11 patients with COPD who were current smokers. In the other study subjects, either it was not possible to induce sputum or samples were not suitable for analysis because of high salivary squamous cells ( $> 30\%$  of total cells). Differential cell counts in induced sputum, expressed as percentage of total nonsquamous cells, are shown in Table 2. Neutrophil cell counts were elevated in patients with COPD who were ex-smokers compared with healthy ex-smokers ( $P < 0.01$ ) and patients with COPD who were current smokers ( $P < 0.04$ ), but not healthy current smokers ( $P = 0.21$ ) (Table 2). Likely due to relative increase in relative percentage of neutrophil cell counts, ex-smokers with COPD had lower macrophage cell counts than healthy ex-smokers ( $P < 0.01$ ), healthy smokers ( $P < 0.03$ ) and current smokers with COPD ( $P < 0.02$ ). No between-group differences were observed in relative percentage of eosinophil, lymphocyte, and bronchial epithelial cell counts.



**Table 2.** Differential cell counts in induced sputum.

	Healthy ex-smokers	Healthy smokers	COPD ex-smokers	COPD current smokers
n	9	6	29	11
Macrophages, %	85.0 (57.5-89.5)	85.5 (58.0-97.3)	47.0 (11.0-80.0) <sup>a,b,c</sup>	75.0 (65.0-92.0)
Neutrophils, %	0 (0-1.5)	12.5 (0-25.0)	26.0 (1.0-82.5) <sup>a,d</sup>	3.0 (0-20.0)
Eosinophils, %	1.0 (0-3.0)	0 (0-1.0)	0.5 (0-2.0)	0 (0-1.0)
Lymphocytes, %	2.0 (0-8.0)	1.0 (1.0-2.0)	1.0 (0-7.0)	1.0 (1.0-12.0)
Bronchial epithelial cells, %	5.0 (2.5-9.5)	1.5 (1.0-3.0)	5.0 (0.25-10.0)	5.0 (1.0-6.0)

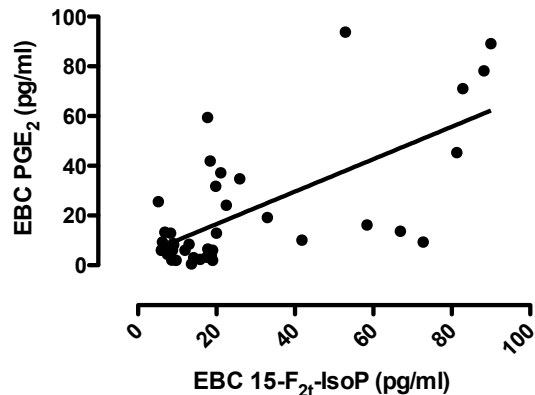
<sup>§</sup>Data are expressed as median and 25%-75% interquartile range.

<sup>a</sup>, P<0.01, compared with healthy ex-smokers; <sup>b</sup>, P<0.03 compared with healthy current smokers;

<sup>c</sup>, P<0.02, compared with COPD current smokers; <sup>d</sup>, P<0.04, compared with COPD current smokers

### Correlations

In patients with COPD who were ex-smokers, there was a correlation between 15-F<sub>2t</sub>-isoprostane and PGE<sub>2</sub> concentrations in EBC (Spearman r = 0.55, P = 0.0002, n = 40) (Figure 6). No correlation was observed between other study outcomes.



**Figure 6.** Correlation between 15-F<sub>2t</sub>-isoprostane and PGE<sub>2</sub> concentrations in EBC in patients with COPD who were ex-smokers (Spearman r = 0.55, P = 0.0002, n = 40).

### Discussion

In our study, we report 1) elevated PGE<sub>2</sub> concentrations in sputum supernatants in both groups of current smokers; 2) elevated 15-F<sub>2t</sub>-isoprostane concentrations in EBC in both groups of patients with

COPD; 3) elevated urinary 15-F<sub>2t</sub>-isoprostane concentrations in both groups of current smokers compared with the respective ex-smoker group; 4) elevated F<sub>E</sub>NO in ex-smokers with COPD compared with both current smoker groups.

Elevated sputum PGE<sub>2</sub> concentrations in both current smoker groups as compared with their ex-smoker counterparts (COPD and healthy control subjects) and higher sputum PGE<sub>2</sub> concentrations in healthy current smokers than in patients with COPD who were ex-smokers suggest that smoking itself can activate cyclo-oxygenase (COX) with subsequent increased airway PGE<sub>2</sub> production independent of COPD. This data interpretation is further supported by in vitro data showing that cigarette smoke extract increases PGE<sub>2</sub> release and COX-2 and PGE<sub>2</sub> receptor expression in neutrophils and alveolar macrophages [11], suggesting that smoking promotes COX-2 induction.

Unlike sputum PGE<sub>2</sub>, EBC PGE<sub>2</sub> was elevated in both current and ex-smokers with COPD who had similar PGE<sub>2</sub> concentrations. These data suggest that EBC PGE<sub>2</sub> levels are more affected by COPD itself than smoking habit and are relatively independent of smoking cessation. This partially different pattern of EBC and sputum PGE<sub>2</sub> concentrations might result from between-technique differences in their capacity of sampling the respiratory system as induced sputum is suitable for central airway sampling [13], whereas EBC has been successfully used for lower airway and alveolar sampling [14]. However, formal studies are required to define the origin of sputum and EBC markers in the respiratory system compartments.

In line with previous studies, we observed elevated oxidative stress, as reflected by 15-F<sub>2t</sub>-isoprostane concentrations in EBC, in both groups of patients with COPD. Similar values in patients with COPD who were either current or ex-smokers might indicate that the increase in EBC 15-F<sub>2t</sub>-isoprostane is relatively independent of smoking cessation suggesting persistent oxidative stress consistent with previous reports [29,30]. In ex-smokers with COPD, the broad range of EBC 15-F<sub>2t</sub>-isoprostane values likely reflect a high inter-individual biological variability. In patients with COPD who were current

smokers, but not in ex-smokers with COPD, elevated 15-F<sub>2t</sub>-isoprostane concentrations in sputum supernatants confirm the increased oxidative stress observed by measuring 15-F<sub>2t</sub>-isoprostane in EBC. In view of the previously mentioned differential sampling capacity of EBC and sputum induction [13,14], the lack of complete concordance of sputum and EBC 15-F<sub>2t</sub>-isoprostane concentrations might suggest that, in current and ex-smokers with COPD, 15-F<sub>2t</sub>-isoprostane is produced, at least partially, at different levels of the respiratory system. In patients with COPD who were ex-smokers, we observed a correlation between EBC concentrations of PGE<sub>2</sub> and 15-F<sub>2t</sub>-isoprostane, a marker of oxidative stress. The role of PGE<sub>2</sub> in the respiratory system is not defined. As PGE<sub>2</sub> can have both pro-inflammatory [1,2] and anti-inflammatory effects [3,4] in the respiratory system, the biological meaning of this correlation is unknown because it could reflect either increased respiratory inflammation which parallels elevated 15-F<sub>2t</sub>-isoprostane or a protective mechanism to counteract oxidative stress.

We observed higher PGE<sub>2</sub> concentrations in sputum supernatants than in EBC (median ratio ranging from 11 to 34 across study groups). In contrast, sputum and EBC 15-F<sub>2t</sub>-isoprostane concentrations were similar. This discrepancy might be explained by differences in biological matrix dilution, biomolecule distribution, technique sampling ability and compartment of biomolecule production within the respiratory system.

We measured 15-F<sub>2t</sub>-isoprostane in EBC, sputum and urine. Urinary 15-F<sub>2t</sub>-isoprostane values were similar to those reported in previous studies [15,27]. The fact that the highest 15-F<sub>2t</sub>-isoprostane values were observed in urine likely reflects systemic production of this marker of oxidative stress and/or its increased stability in this biological matrix [5].

The increase in F<sub>E</sub>NO values in ex-smokers with COPD compared with healthy ex-smokers, which is very close to statistical significance (P = 0.0546), might suggest COPD-associated airway inflammation in this group of patients. Lower F<sub>E</sub>NO concentrations in both groups of smokers possibly result from smoke-induced increase in production of reactive oxygen species which might convert NO

into peroxynitrite, thus, reducing  $F_{E}NO$  levels [31]. In addition to that, cigarette smoke reduces inducible nitric oxide synthase (NOS) activity in lung epithelial cells [32] and endothelial NOS in pulmonary artery endothelial cells [33].

Relative percentage of sputum neutrophil cell counts was increased in ex-smokers with COPD compared with healthy ex-smokers in line with COPD-induced airway inflammation. Higher sputum neutrophils in ex-smokers with COPD than in current smokers with COPD is difficult to explain and might be related to between-group differences in smoking history and/or airway/lung bacteriological profiles. However, sputum bacteriological analysis was not performed.

Our study has strengths. All the techniques used for assessing inflammation/oxidative stress are non-invasive, except sputum induction, which is semi-invasive; the methodology used is reliable as  $PGE_2$  and 15- $F_{2t}$ -isoprostane were measured with techniques previously validated by GC/MS or HPLC [7,21,22], which are analytical gold standard, whereas EBC sampling [18],  $F_{E}NO$  measurement [19,15], and sputum induction and analysis [24-26] were performed according to international guidelines; in study groups, the same panel of outcomes, including systemic (urinary 15- $F_{2t}$ -isoprostane) and respiratory (sputum and EBC  $PGE_2$  and 15- $F_{2t}$ -isoprostane,  $F_{E}NO$ , sputum cell counts) markers, was measured making it possible to compare inflammation/oxidative stress at the systemic and local level in the same cohort; measurement of  $PGE_2$  in EBC and sputum and 15- $F_{2t}$ -isoprostane in EBC, sputum and urine, enables concentration comparison of the same outcome measures in various biological matrices; the effect of smoking habit and COPD itself can be studied due to the inclusion of healthy control groups (current smokers and ex-smokers).

However, our study has some limitations. A possible effect of age on inflammatory outcome measures cannot be excluded as healthy current smokers were younger than other study groups. Ex-smokers with COPD had a heavier smoking history than healthy current smokers based on pack-years and more severe airway limitation than current smokers with COPD based on  $FEV_1$  and  $FEV_1/FVC$  values.

PGE<sub>2</sub> concentrations were detected in all EBC samples, although in some cases, particularly in healthy ex-smokers, were close to the analytical sensitivity where the variability is higher. Likewise, sputum 15-F<sub>2t</sub>-isoprostane data should be interpreted cautiously as concentrations were undetectable in 6 ex-smokers with COPD and 2 healthy ex-smokers and, in some cases, were close to the analytical detection limit. This is a relatively small study which requires larger confirmatory studies.

## **Conclusion**

The biological meaning of the inflammatory markers measured in this study depends on type of marker and biological matrix in which is measured. We propose a holistic, integrated, approach to the patient with COPD based on measurement of exhaled and non-exhaled non-invasive markers of respiratory and systemic inflammation/oxidative stress that combines functional, cellular and molecular outcomes. This strategy has potential and therapeutic implications and might translate into a more complete characterization of individual patients with COPD. However, larger studies and external validation are required to clarify the clinical utility of this approach.

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