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Gene and Protein Expression of Fibronectin and Tenascin-C in Lung Samples from COPD Patients

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Abstract
Purpose Fibronectin (Fn) and tenascin-C (TnC) are two extracellular matrix proteins associated with remodeling changes. Fn and TnC gene and protein expression in lung tissue, including their predominant location in bronchial and pulmonary artery structures, have not yet been fully evaluated. The aim of the present study was to assess: (1) gene expression of Fn and TnC in lung samples from chronic obstructive pulmonary disease (COPD) and non-COPD subjects; and (2) protein content and location of Fn and TnC in both groups.
Methods Consecutive subjects requiring lung resection due to lung cancer surgery were included. Lung specimens were examined for gene expression by quantitative real-time PCR (values expressed as fold change ratio). The analysis of their protein content and location was performed by western blot and immunohistochemical studies, respectively. Patients were divided into two cohorts according to COPD status.
Results A total of 41 patients (20 with COPD and 21 without COPD) were included. An enhanced Fn gene expression was observed in the COPD group compared to the non-COPD group (4.73 ± 0.54 vs. 2.65 ± 0.57; $P = 0.012$), whereas no differences in gene TnC expression were observed (2.91 ± 0.44 vs. 2.60 ± 0.48; $P = 0.633$). No differences in lung protein content and location were found between groups. Immunohistochemical evaluation showed a predominantly vascular and bronchial location of Fn and TnC in both groups.
Conclusions An enhanced lung gene expression of Fn was observed in COPD subjects compared to non-COPD subjects. No differences were found in Fn protein expression or in TnC gene or protein expression among groups.

Keywords Gene expression · Remodeling · Vascular · Extracellular matrix proteins

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive pulmonary changes with extrapulmonary consequences, as well as by a state of persistent local inflammation in most patients [1–4]. This ongoing chronic inflammation can trigger remodeling processes of pulmonary structures mediated by cytokines and growth factors [5–7]. The presence of vascular remodeling has been described from the early stages of the disease, even in patients with preserved lung function [8]. Of note, pulmonary vascular remodeling plays an important role in the subsequent development of pulmonary hypertension in COPD [9].

Some of the extracellular matrix (ECM) proteins, such as fibronectin (Fn) and tenascin-C (TnC), have an active role in the inflammatory processes, contributing to re-building and repairing human tissues after injury [10–12]. However, there is little information available about the Fn and TnC gene re-expression under pathological conditions such as COPD. An increased gene expression of Fn at early stages of COPD and a progressive down-regulation in advanced stages of the disease has been described in lung samples [13]. Other immunohistochemical studies have observed an association between an increased TnC and Fn protein expression with the bronchial remodeling changes observed in asthma and COPD patients [14–16]. Noteworthy, these previous experiences were focused on describing only bronchial structures and lung parenchyma, whereas the vascular component of the lung (pulmonary arteries) was not analyzed in these studies [14–16].

To date, to the best of our knowledge, no study has evaluated simultaneously the pre- (gene) and post-transcriptional (protein) lung expression of Fn and TnC in samples from COPD patients, comparing the results with those from a cohort of patients without COPD. The objective of the present investigation was twofold: (1) to evaluate gene expression of Fn and TnC in lung samples from COPD and non-COPD subjects; and (2) to assess protein content and location of Fn and TnC in both groups.

Materials and Methods

Study Subjects

This was a prospective investigation conducted in consecutive subjects who required lung resection for treatment of primary lung cancer. All patients had history of current or former smoking habit of more than 10 pack-years, and were classified as (1) COPD patients and (2) non-COPD patients (criteria from the GOLD guidelines [1]). Exclusion criteria were the presence of any other pulmonary disease different to COPD and prior chemotherapy or radiotherapy treatment. All subjects underwent a post-bronchodilator spirometry and a test for determining diffusion lung capacity for carbon monoxide (DL_{CO}) prior to surgery. The study protocol was approved by the local ethics committee and performed in accordance with the Declaration of Helsinki. All patients provided written informed consent.

Sample Collection

Lung specimens were obtained from pieces of lung resection, as far away as possible from the tumor, and were processed immediately. A microscopic evaluation to confirm the absence of neoplastic cells was performed on each tissue sample before being included in the analysis.

Quantitative Real-Time-PCR

Quantitative real-time-PCR (qRT-PCR) was performed to determine the lung gene expression of Fn and TnC. In particular, the Fn isoform evaluated contains the type III extra domain A (ED-A), which is a specific marker involved in blood vessel morphogenesis [12], a crucial mechanism in remodeling processes [17]. In brief, tissue sections were stored in RNA stabilization reagent (RNA*later*, Qiagen) at −80 °C and RNA isolation was performed using the Trizol method. TaqMan Universal PCR Master Mix (Applied Biosystems) was used to amplify the cDNA. Using the $2^{\Delta\Delta C_t}$ method, the mRNA expression of the target genes was normalized for the expression of housekeeping genes (18S rRNA and DNA-directed RNA polymerase II). A TaqMan assay was designed for ED-A variant of Fn, using the following sequence: forward primer, AGGACTGGCATTCA CTGATGTG; reverse primer, GTGGGCTTTCCCAAGC AA; and probe, ATGTCGATTCCATCAAA. Commercial inventoried TaqMan assays were used for TnC (Applied Biosystems TaqMan Assay, Hs01115665_m1) and 18S (Applied Biosystems Taqman Assay, Hs99999901_s1). A common calibrator for each plate was used. Data are reported as a relative quantification (fold change ratio) of mRNA of Fn and TnC from each sample.

Immunohistochemistry

The Fn and TnC location in lung tissue was examined by immunohistochemistry methods. Serial sections of paraffin-embedded lung tissue were immunostained overnight with monoclonal antibodies against Fn (ED-A, 1/400, AB6328; Abcam) and TnC (1/300, AB3970; Abcam) using the avidin–biotin complex/peroxidase (Vectastain Elite ABC kit, Vector Laboratories). Immunoreaction analysis was evaluated in: (1) the pulmonary muscular arteries (100–500 μm): endothelial cells and smooth muscles cells (SMCs); (2) the bronchial structures: epithelial cells layer, sub-epithelial baseline membrane (SEBM), and airway smooth muscle cells (ASMCs); and (3) lung parenchyma. The pulmonary arteries and bronchial structures with positive immunoreaction were expressed as a percentage of the total arteries and bronchus examined, respectively. Lung parenchyma with positive immunoreaction was expressed as the percentage of stained area. The intensity of the positive immunoreaction was graded as 1, 2, and 3 meaning mild, moderate, and intense staining, respectively. An average grading score was computed for arteries and bronchial structures in each subject.

Western Blot Assays

The protein content of lung tissue was examined by western blot analysis. Previously, the Lowry method was used to quantify the protein extracts. In short, an amount of 100 μg of protein was denatured and placed in a sodium dodecyl sulphate polyacrylamide gel (7 %) for electrophoresis to be subsequently transferred to nitrocellulose membrane (Bio-Rad and Mini protean II). Overnight incubation at 4 °C was performed with monoclonal primary antibodies against ED-A Fn (1/300 dilution, AB6328, Abcam) and TnC (1/150 dilution, AB3970; Abcam). After 1-h incubation with secondary antibody (Ab. anti-IgG1; R&D Systems), specific immunoreactivity was detected with the avidin–biotin–peroxidase complex method (Vector). Protein bands were visualized by enhanced chemiluminescence (Supersignal Western, Pierce) and digitized using the Image Reader LAS-3000 (Fuji). Multi-gauge v1.3 Software was used to quantify the densitometry of the protein band intensity. The ratio of the band intensity (target protein/β-actin, A1978; Sigma) was used as a measure of the protein content in each sample.

Study Endpoints and Sample Size Calculation

The primary endpoint of this study was the fold change ratio of lung gene expression (ED-A Fn or TnC) in COPD patients compared to non-COPD patients. Assuming a standard deviation of 2.0, a sample size of 18 subjects per group was needed to detect a difference between groups of the usual arbitrary fold change cutoff of 2 (minimal significant difference for gene expression analysis) [18]; with 85 % power and a two-tailed α-value less than 0.05. Considering an approximate 17 % dropout rate (e.g., insufficient RNA obtained for PCR), inclusion of 44 subjects was allowed to ensure that gene expression data from 18 patients were available for analysis. Secondary endpoints included between-group comparisons for: (1) percentage and intensity of positive immunoreaction for Fn and TnC in arterial and bronchial structures, and (2) protein content of Fn and TnC in lung tissue.

Statistical Analysis

For baseline characteristics, continuous variables were expressed as mean ± SD or median and interquartile range whether a normal distribution was assumed or not (Kolmogorov–Smirnov test), respectively. Comparisons of continuous variables were performed with Student's *t* test or Mann–Whitney's *U* test as appropriate, while qualitative variables were compared with Chi square test or Fisher's exact test (any expected value <5). An ANOVA method with a general linear model was used to evaluate the primary endpoint and all other between-group comparisons. Adjusted analyses were performed with an ANCOVA method, using as covariates unbalanced demographic or clinical variables ($P < 0.10$) or those variables considered relevant. Finally, covariates included were age, gender, current smoking habit, and use of inhaled corticosteroids and statins. Since active smoking and the use of inhaled corticosteroids have been associated with regulation of Fn and/or TnC expression [19–23], exploratory stratified analyses were performed in order to assess the possible effect of these variables on gene expression. A two-tailed P value of <0.05 was considered to indicate a statistically significant difference. Results are reported as least squares mean (LSM) ± standard error of the mean (SEM) for the above detailed analyses. Statistical analysis was performed using the PASW Statistics v18.0 software (SPSS Inc., Chicago, IL).

Results

Consecutive samples from 44 patients undergoing lung resection surgery were included in the study, of which three of them were discarded due to poor quality of the sample or insufficient RNA obtained. Therefore, 41 patients were included in the present analysis, 20 COPD subjects (12 former smokers and 8 current smokers), and 21 non-COPD patients (12 former smokers and 9 current smokers). There were no significant differences in baseline characteristics (Table 1) between groups, except for gender and age.

Gene Expression

An enhanced Fn gene expression was observed in the COPD group compared to the non-COPD group (4.73 ± 0.54 vs. 2.65 ± 0.57; unadjusted P value = 0.012 and adjusted P value = 0.030). Conversely, no difference was observed for TnC gene expression (2.91 ± 0.44 vs. 2.60 ± 0.48; unadjusted P value = 0.633 and adjusted P value = 0.669). In addition to COPD status, a current smoking habit was the only covariate significantly associated with Fn gene expression in the adjusted analysis ($P = 0.019$), observing an inverse relationship of current smoking habit with Fn expression. In the stratified analysis considering both COPD and smoking status, a higher Fn gene expression was found in the subset of patients with COPD who were former smokers (Fig. 1a), which was statistically significant when compared to COPD current smokers ($P = 0.032$). No differences were observed in TnC gene expression when evaluating smoking habit (Fig. 1b) as well as no differences in Fn or TnC gene expression were observed regarding the use of ICS in COPD patients (data not shown).

Table 1 Baseline characteristics and lung function parameters of the study groups

Characteristic	Non-COPD ($n = 21$)	COPD ($n = 20$)	P value
Male gender, n (%)	16 (76.2)	20 (100)	0.048
Age (years)[a]	61.1 ± 9.13	67.5 ± 6.10	0.011
BMI (kg/m^2)	25.1 [23.4–27.4]	27.9 [24.2–29.6]	0.060
Pack-years[a]	37.4 ± 20.3	46.1 ± 20.3	0.182
Current smoking, n (%)	9 (42.9)	8 (40.0)	0.853
Years stopped smoking[a]	11.6 ± 4.1	14.3 ± 2.3	0.323
COPD stage I/II, n (%)	–	7 (35)/13(65)	–
Pulmonary hypertension, n (%)	0 (0)	2 (10.0)	0.487
Diabetes Mellitus, n (%)	3 (14.3)	3 (15.0)	1.000
Dyslipidemia, n (%)	7 (33.3)	10 (50.0)	0.279
Systemic hypertension, n (%)	10 (47.6)	12 (60.0)	0.427
Coronary arterial disease, n (%)	1 (4.8)	2 (10.0)	0.606
Statins, n (%)	7 (33.3)	9 (45.0)	0.444
ICS, n (%)	0(0)	8 (40.0)	0.001
FVC, % predicted	94.0 [80.1–106.7]	95.1[81.4–114.1]	0.896
FEV_1, % predicted	91.0 [84.1–99.9]	73.2 [62.9–91.1]	0.003
FEV_1/FVC, %	76.0 [72.7–79.2]	63.1 [57.7–67.6]	<0.001
DL_{CO}, % predicted	82.6 [73.6–88.0]	75.9 [59.8–93.9]	0.360

Data reported as mean ± SD or median [IQR]

BMI body mass index, *ICS* inhaled corticosteroids, *FVC* forced vital capacity, *FEV_1* forced expiratory volume in one second, *DL_{CO}* diffusion lung capacity for carbon monoxide

P value of <0.05 was considered statistically significant

[a] Variables with a normal distribution

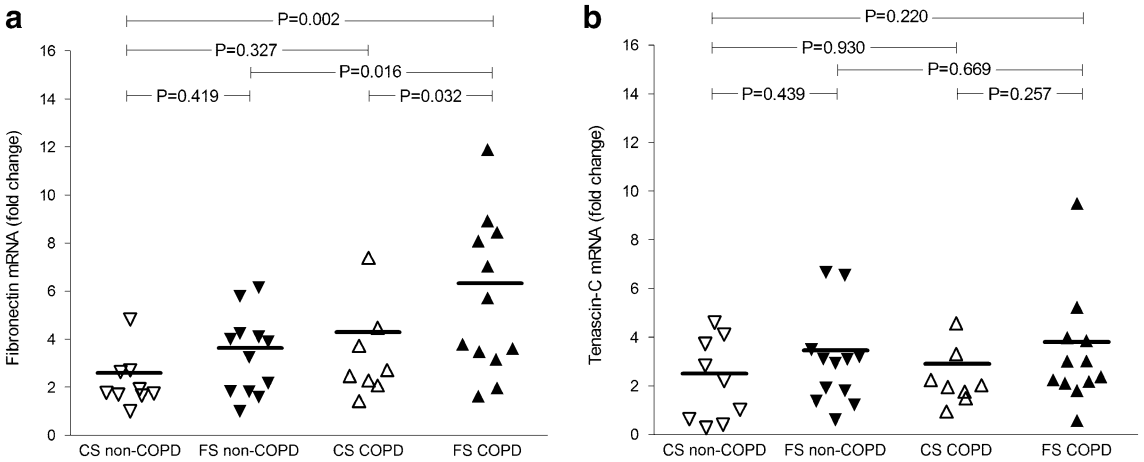

Fig. 1 Lung gene expression according to smoking habit and COPD and non-COPD groups. Individual data of the mRNA expression (fold change) of fibronectin (**a**) and tenascin-C (**b**) in lung tissue, according to groups and smoking habit. *Horizontal bars* indicate least squares mean values. *CS* current smokers, *FS* former smokers

Protein Location

Immunoreaction results are summarized in Table 2. In pulmonary arteries, Fn was predominantly expressed in endothelial cells and SMCs, whereas TnC mainly in SMCs. In bronchial structures, positivity for Fn and TnC was predominantly observed in the cytoplasm of epithelial cells; with mild or no expression in SEBM or ASMCs. A positive immunoreaction for Fn and TnC was also observed on alveolar epithelium and capillaries of the alveolar wall, constituting more than 60 % of stained area of the lung parenchyma (66.4 ± 7.1 % and 70.1 ± 12.2 % for Fn and TnC, respectively). No differences in the percentage of positivity or the intensity score between COPD and non-

Table 2 Immunohistochemical lung expression of ED-A fibronectin and tenascin-C by groups

Characteristic	ED-A fibronectin			Tenascin-C		
	Non-COPD	COPD	*P* value	Non-COPD	COPD	*P* value
N° arteries by patient[a]	16.4 ± 5.6	15.9 ± 6.8	0.821	14.0 ± 6.3	14.6 ± 7.2	0.843
Positive immunoreaction in ECs (%)	100 [97.1–100]	100 [97.2–100]	0.976	0 [0–27.6]	0 [0–14.1]	0.796
Positive immunoreaction in SMCs (%)	66.7 [27.9–96.3]	78.6 [59–87.5]	0.376	42.6 [16.8–67.5]	34.3 [18.1–64.4]	0.790
Intensity of the immunoreaction in ECs	1.2 [1–2]	1.4 [1–1.8]	0.738	1 [0.5–1]	1 [1–1.1]	0.429
Intensity of the immunoreaction in SMCs	1 [1–1.9]	1 [1–1.3]	0.556	1 [1–1.2]	1 [1–1.4]	0.931
N° bronchial structure by patient[a]	5.6 ± 3.2	5.9 ± 4.2	0.821	5.8 ± 2.4	5.4 ± 3.8	0.835
Positive immunoreaction in BE (%)	83.3 [43.7–100]	100 [92.5–100]	0.129	80 [33.3–100]	100 [75–100]	0.393
Positive immunoreaction in SEBM (%)	12.5 [0–16.6]	12.9 [0–18.6]	0.808	20 [0–100]	37.5 [0–70.8]	0.877
Positive immunoreaction in ASMc (%)	12.5 [0–27.1]	10 [0–37.1]	1.0	14.3[0–20]	10 [0–14.6]	0.311
Intensity of the immunoreaction in BE	1 [1–1]	1 [1–1.1]	0.508	1 [1–1]	1 [1–1]	0.831
Intensity of the immunoreaction SEBM	1 [1–1.1]	1 [1–1.3]	0.662	1 [1–1.5]	1 [0–1]	0.400
Intensity of the immunoreaction ASMc	1 [1–1.2]	1 [1–1.3]	1.0	1 [0–1]	1 [0–1]	0.244
Lung parenchyma						
Positive immunoreaction (%)	69.0 [59.1–72]	62.0 [59.0–72.1]	0.403	70.0 [60.5–79.5]	76.5 [69.3–79.0]	0.976
Intensity of the immunoreaction	2 [1–3]	3 [1.5–3]	0.284	1 [1–2]	1 [1–2]	0.670

Data reported as median [IQR] or mean ± SD

ED-A extra domain A of fibronectin, *ECs* endothelial cells, *SMCs* smooth muscle cells, *BE* bronchial epithelium, *SEBM* sub-epithelial baseline membrane, *ASMc* airway smooth muscle cells

A 2-tailed probability value of $P < 0.05$ was considered to be statistically significant

[a] Variables with a normal distribution.

The pulmonary arteries and bronchial structures with positive immunoreaction were expressed as a percentage of the total arteries and bronchus examined, respectively. Lung parenchyma with positive immunoreaction is expressed as the percentage of stained area. The intensity of the positive immunoreaction was additionally graded as 1, 2, and 3 meaning mild, moderate, and intense staining, respectively

COPD patients were observed at any location. Representative photomicrographs of immunohistochemical expression of Fn and TnC in lung tissue from COPD and non-COPD subjects are represented in Figs. 2 and 3, respectively.

Protein Content

Western blot analysis was performed to confirm the presence of Fn and TnC protein content in total lung tissue, previously assessed in the immunohistochemical analyses described above. No differences were observed for the protein signal of Fn (0.89 ± 0.14 vs. 0.80 ± 0.16; $P = 0.661$) and TnC (1.27 ± 0.19 vs. 1.20 ± 0.19; $P = 0.806$) between COPD and non-COPD subjects in the densitometric analysis (Fig. 4) of the protein bands.

Discussion

In the present investigation, we assessed both the gene and the protein expression of two relevant ECM components, Fn and TnC, in the lung tissue of a group of patients with mild or moderate COPD and a group of non-COPD subjects (smokers with normal lung function). Previous evidence for the gene re-expression of these two remodeling proteins in patients with COPD is scarce. In particular, Gosselink and colleagues observed a decreased expression of Fn, among other remodeling proteins, inversely related to FEV_1, which suggests a progressive down-regulation of Fn gene expression associated with lung function worsening and, thus, progression of the disease [13]. In the present study, an up-regulation of Fn gene expression was observed in total lung tissue extracted from COPD patients compared to non-COPD subjects. Of note, all patients included in the analysis had a mild or moderate form of the disease and a preserved DL_{CO}, which cannot rule out the presence of mild emphysema but denotes a lack of significant parenchyma destruction and that these patients had not developed severe emphysema. These findings might support the hypothesis of an initial enhanced gene expression of some ECM proteins such as Fn at early stages of COPD, being involved in initial vascular and airway remodeling changes, which decreases with severity of emphysema and progression of the disease, and is overall in line with results from prior investigations [13, 24]. On the contrary, a significant increase in the gene

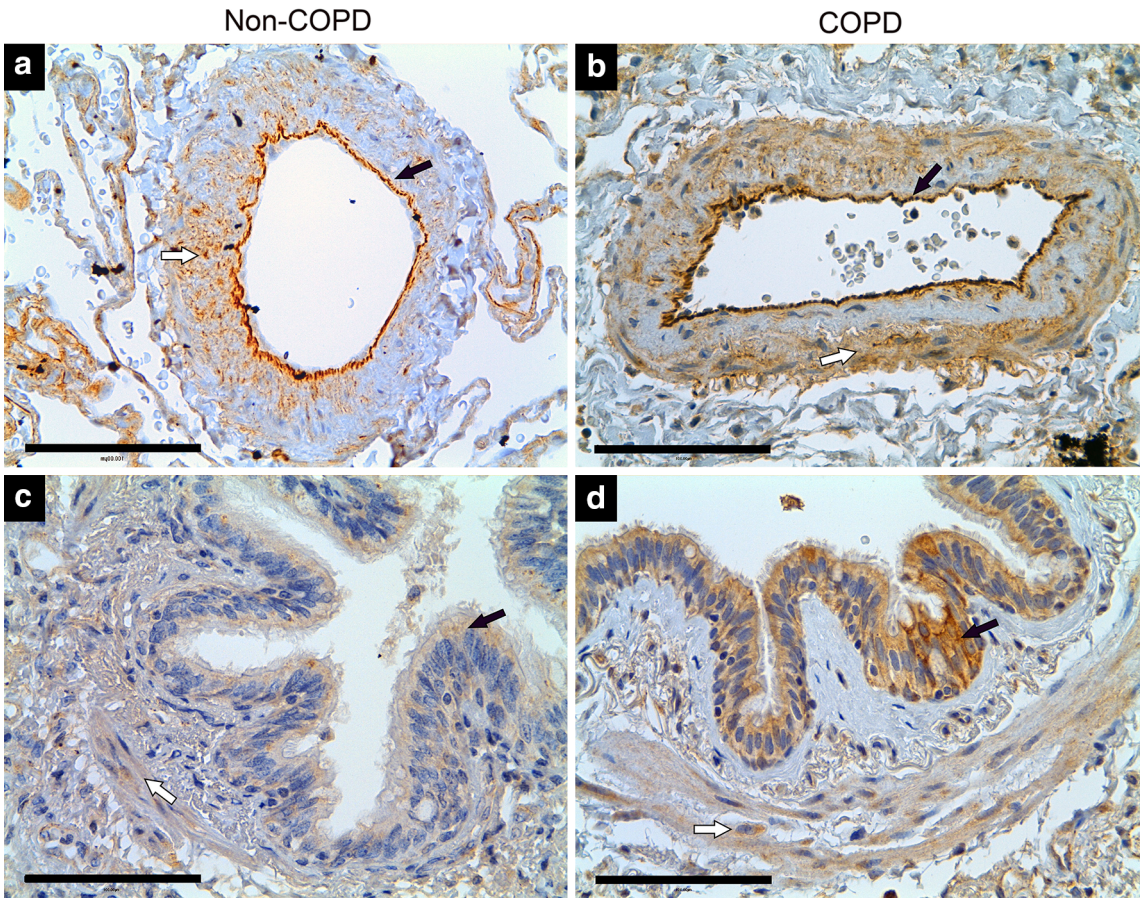

Fig. 2 Representative photomicrographs of immunohistochemical expression of ED-A fibronectin in lung tissue from non-COPD and COPD subjects. Image **a** represents a pulmonary muscular artery from a non-COPD subject, which has a positive immunoreaction in endothelial cells layer (*black arrow*) and a mild expression in smooth muscles cells from medial layer (*white arrow*). Image **b** shows a pulmonary artery from a COPD subject with a positive immunoreaction in endothelium (*black arrow*); and also in the medial layer (*white arrow*). Images **c** and **d** represent bronchial structures from a non-COPD and COPD subject, respectively, with a positive immunoreaction in the cytoplasm from epithelial cells (*black arrows*), and a mild immunoreaction in airways smooth muscles cells (*white arrows*). *Scale bar* 100 μm

expression of TnC, which has been associated with remodeling processes in several settings [11, 12, 25], was not seen in COPD patients compared to non-COPD subjects in the present investigation. This could be attributed to a differential gene expression of TnC that may depend on the underlying disease. In fact, a recent investigation by Hoffmann and colleagues observed a distinct gene expression pattern of TnC in patients with pulmonary hypertension, comparing subjects with COPD to individuals with idiopathic pulmonary fibrosis, which suggests that pulmonary arterial remodeling is caused by different molecular mechanisms that may vary depending on the specific pulmonary disease [26]. Whether these findings might overall suggest a predominant role of Fn in the initial changes leading to remodeling of arteries in COPD patients warrants further investigation.

Other factors such as smoking habit and the use of corticosteroids have been associated with regulation of gene and protein expression of several ECM components under in vitro conditions (fibroblasts culture) or in tissue samples [19–23, 27]. In particular, it has been reported in a mechanistic study that the volatile components of cigarette smoke in acute exposures inhibit lung fibroblast proliferation and decrease the production of some ECM proteins such as Fn [19], which could be of relevance in terms of progression of COPD. This is in line with the findings of the present investigation, since we observed a significant decrease of Fn gene expression associated with current smoking habit in the overall population of the study, which was statistically significant in the subset of patients with COPD. To the best of our knowledge, the association of smoking habit with TnC gene expression in COPD or non-COPD subjects has not been evaluated previously, and we failed to find any difference between groups in the present investigation. Despite the fact that there are to date very few data available, it has been suggested that corticosteroids may modulate extracellular matrix composition in

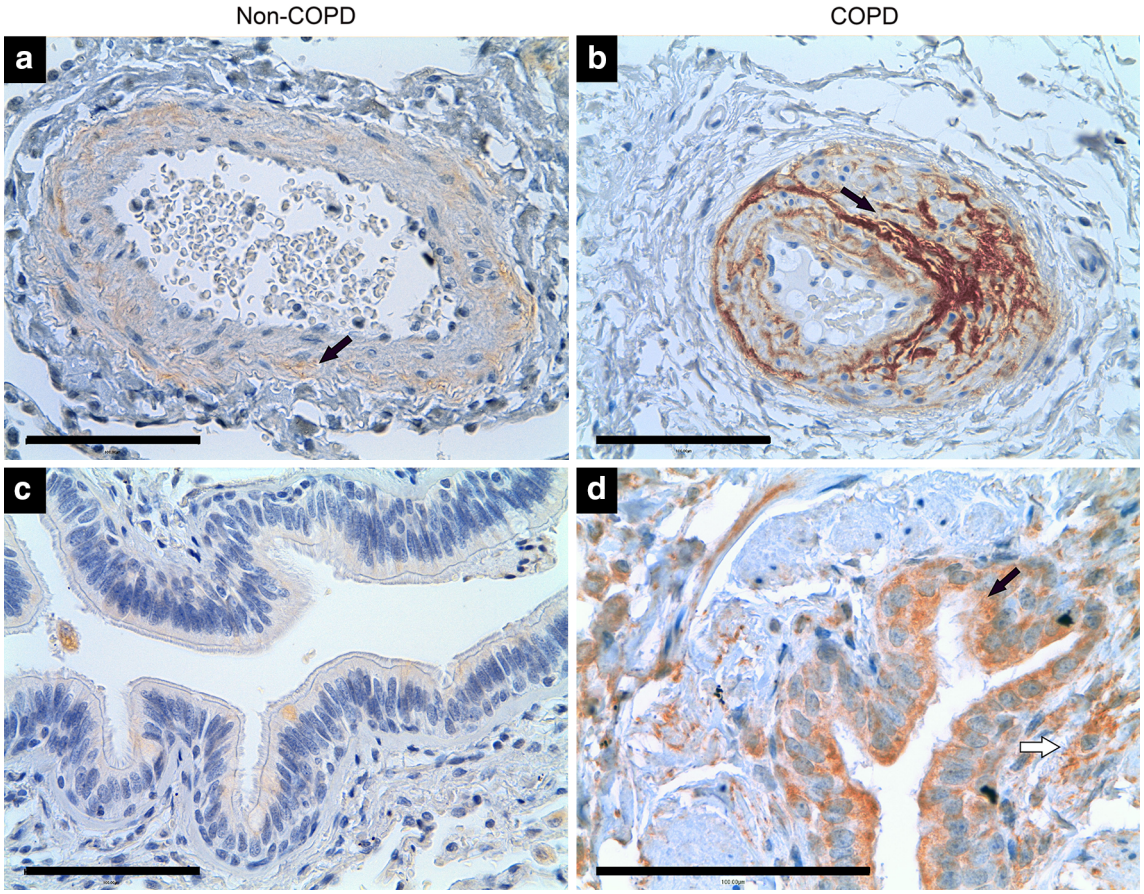

Fig. 3 Representative photomicrographs of immunohistochemical expression of tenascin-C in lung tissue from non-COPD and COPD subjects. Image **a** represents a pulmonary muscular artery from a non-COPD subject, which has a negative immunoreaction at endothelial cells and a mild expression in the medial layer (*black arrow*). Image **b** shows a remodeled pulmonary artery from a COPD subject with a negative immunoreaction at endothelial cells and an intense positive immunoreaction at medial layer (*black arrow*). Images **c** and **d** represent bronchial structures from a non-COPD and COPD subject, respectively, with a negative immunoreaction (**c**) at epithelial cells and airways smooth muscles cells in non-COPD subject. A positive immunoreaction (**d**) is represented in the cytoplasm from epithelial cells (*black arrow*) and airways smooth muscles cells (*white arrow*). *Scale bar* 100 μm

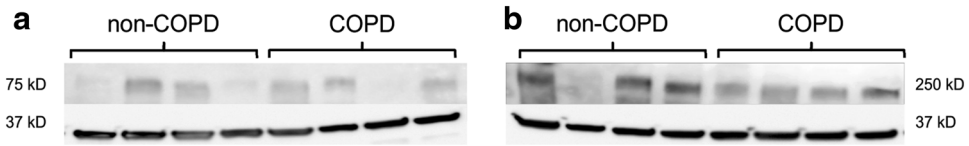

Fig. 4 Protein content of fibronectin and tenascin-C in lung tissue according to groups. **a** Western blot analysis for fibronectin in lung tissue of non-COPD subjects and COPD subjects. Bands at 75 and 37 kDa are consistent with size of fibronectin and β-actin, respectively. **b** Western blot analysis for tenascin-C in lung tissue of non-COPD subjects and COPD subjects. Bands at 250 and 37 kDa are consistent with the size of tenascin-C and β-actin, respectively

lung diseases [21–23]. A single in vitro investigation has observed that a corticosteroid (fluticasone) may decrease TnC and ED-A Fn expression in cultures of human fibroblasts [21]. Using a different approach, we did not observe a different pattern of gene expression in COPD subjects undergoing inhaled corticosteroid treatment in the present investigation.

A novel finding of the present study is the evidence of protein expression of Fn and TnC in the pulmonary arteries from COPD subjects and non-COPD smokers, as these proteins are not usually detected in normal tissue [12, 28]. TnC expression at the intima and media layer of remodeled pulmonary vessels from idiopathic pulmonary fibrosis subjects with pulmonary hypertension has been recently reported [26]. Interestingly, an up-regulation of TnC gene expression was observed in these patients when compared to COPD subjects [26]. In addition, a higher protein expression of Fn and TnC has been associated to a gradient

for progressive vascular remodeling processes in patients with pulmonary hypertension due to congenital heart diseases [29]. Overall, these data support the idea that these ECM proteins could play a role in early vascular remodeling processes, even in smokers with normal lung function, although the molecular mechanisms may vary according to underlying pathologies.

We acknowledge the inherent limitations of this investigation due to its observational design and a small sample size, as the study was powered to detect differences in the primary end point but not for multiple comparisons. In addition, the lack of a control group (never smokers) makes it difficult to draw definitive conclusions about the influence of chronic tobacco exposure in this setting. However, we included subjects with past and current smoking habit, which provide interesting inputs regarding the influence of acute smoke exposure in gene expression.

In summary, an enhanced lung gene expression of Fn was observed in COPD subjects compared to non-COPD subjects, with differences in the Fn expression under smoking conditions. Furthermore, there is a lung vascular protein expression of Fn and TnC, even in smokers.

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Conflict of interest The authors declare that they have no others conflicts of interest.

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