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Extracellular matrix composition in COPD

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ABSTRACT: Extracellular matrix (ECM) composition has an important role in determining airway structure. We postulated that ECM lung composition of chronic obstructive pulmonary disease (COPD) patients differs from that observed in smoking and nonsmoking subjects without airflow obstruction.

We determined the fractional areas of elastic fibres, type-I, -III and -IV collagen, versican, decorin, biglycan, lumican, fibronectin and tenascin in different compartments of the large and small airways and lung parenchyma in 26 COPD patients, 26 smokers without COPD and 16 nonsmoking control subjects.

The fractional area of elastic fibres was higher in non-obstructed smokers than in COPD and nonsmoking controls, in all lung compartments. Type-I collagen fractional area was lower in the large and small airways of COPD patients and in the small airways of non-obstructed smokers than in nonsmokers. Compared with nonsmokers, COPD patients had lower versican fractional area in the parenchyma, higher fibronectin fractional area in small airways and higher tenascin fractional area in large and small airways compartments. In COPD patients, significant correlations were found between elastic fibres and fibronectin and lung function parameters.

Alterations of the major ECM components are widespread in all lung compartments of patients with COPD and may contribute to persistent airflow obstruction.

KEYWORDS: Chronic obstructive pulmonary disease, cigarette smoking, extracellular matrix, pathology, respiratory function tests

Chronic obstructive pulmonary disease (COPD) is a leading cause of mortality and morbidity worldwide. Its burden is still underestimated as COPD is under-diagnosed and under treated in high- and low-income countries, mainly in the mild stages of the disease [1–3]. Smoking is the most important risk factor for the development of COPD. It has been proposed that the chronic cigarette-induced inflammation is associated with the development of structural changes in the lungs of susceptible smokers, which contribute to progressive airflow limitation [4, 5].

The major lung extracellular matrix (ECM) components are collagens, elastic fibres, proteoglycans, fibronectin and tenascin [6, 7]. Previous studies have reported a decrease of elastin [8–10] and proteoglycans [11] and an increase of total collagen content in the alveoli of COPD patients [12]. Few studies have assessed ECM composition at different levels of the airways and lung parenchyma [13, 14].

Collagens are the most abundant components of the lung interstitium and, particularly the fibrillar type-I and -III collagens, are important in maintaining the lung architecture. Type-IV collagen is the main constituent of basement membranes and the most abundant non-fibrillar collagen in the lungs [15, 16].

Proteoglycans are macromolecules composed of a protein core and glycosaminoglycan side chains that are involved in maintaining the assembly of collagen fibrils, water balance and cell adhesion and migration [17, 18]. Little is known about the pattern of proteoglycans deposition in the lungs of COPD patients. So far, studies described alterations of versican and decorin in the distal lung [10, 11].

Tenascin and fibronectin are altered in ongoing tissue injury, regulating important cell properties and inflammatory cell chemotaxis [19]. There are few studies analysing the expression of tenascin

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and fibronectin in COPD patients [13, 20, 21], but no study has addressed these proteins in all lung compartments.

We hypothesised that the composition of ECM is different in the large airways, small airways and lung parenchyma and between patients with COPD and smokers and nonsmokers with normal lung function. Furthermore, we hypothesised that such differences contribute to lung function impairment in COPD.

Therefore, our aim was to quantify the composition of several ECM components (elastic fibres, type-I, -III and -IV collagen, versican, decorin, biglycan, lumican, fibronectin and tenascin) in all lung compartments of patients with COPD, in relation to cigarette smoking and lung function.

METHODS

This study was approved by the review board of the São Paulo University Medical School and A.C. Camargo Hospital (both São Paulo, Brazil), Leiden University Medical Centre (Leiden, the Netherlands) and Palermo University (Palermo, Italy). All subjects provided written informed consent.

Subjects

We analysed lung tissue collected from 68 patients undergoing lung resection surgery for primary or metastatic lung tumours from 2001 to 2007.

Information including demographic data, medical and smoking history, medications and pre-operative lung function was obtained from the patients' hospital charts. Patients with a diagnosis of asthma, bronchiectasis, infectious diseases, α_1-antitrypsin deficiency or interstitial lung disease were not included.

Patients were classified as follows. 1) Nonsmokers (NS, n=16); never-smokers, forced expiratory volume in 1 s (FEV_1) $\geqslant$80% predicted and FEV_1/forced vital capacity (FVC) $\geqslant$70%. 2) Non-obstructed smokers (NOS, n=26): current and/or ex-smokers (quit for $\geqslant$1 month) with normal lung function (FEV_1 $\geqslant$80% pred and FEV_1/FVC $\geqslant$70%). 3) COPD (n=26): current and/or ex-smokers (quit for $\geqslant$1 month) with COPD (FEV_1/FVC $<$70%). Post-bronchodilator values were available in 15 COPD patients (five Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I, mild, nine GOLD stage II, moderate, and one GOLD stage III, severe, [1]), and all showed $<$12% improvement compared with the pre-bronchodilator value.

Tissue processing

Two to four blocks of peripheral parenchyma and one or two blocks of central airways remote from the tumour were obtained in most cases. In general, less tissue was available from central areas because of tumour proximity or surgical borders. Fragments were fixed in 10% buffered formalin for 24 h, processed and paraffin embedded. 4-μm thick sections were stained with haematoxylin–eosin for initial analysis. We excluded cases showing fibrotic disorders, neoplastic tissue and post-stenotic pneumonia.

Histochemistry

For identification of elastic fibres, the Weigert's Resorcin–Fuchsin technique with oxidation was used [22].

Immunohistochemistry

Antigen retrieval and primary antibodies are shown in table 1S in the online supplementary material. Details of the immunohistochemical techniques are described in the online supplementary material.

Morphological analysis

Two large (epithelial basement membrane perimeter $>$6 mm) airways and three small ($\leqslant$6 mm) airways cut in a transverse section, and 10 peribronchiolar (the site of alveolar attachments) and 10 distal alveolar segments (alveolar septa positioned at least 1×100 field from any small airway border) were analysed for all subjects [23].

The airway walls were subdivided into the inner layer, comprising the region between the epithelium and the internal smooth muscle border, the smooth muscle layer and the outer layer, located between the external smooth muscle border and the external limit of the airway, *i.e.* the alveolar parenchyma (fig. 1S in the online supplementary material).

In large airways, type-IV collagen and tenascin mainly stained the subepithelial region of the bronchial epithelial layer and the walls of blood vessels. To avoid including the type-IV collagen and tenascin present in blood vessels, we analysed only subepithelial areas in the large airways. These were defined as a region of 12 μm below the epithelium. We further analysed the muscle layer of the large airways, the inner and muscle layer of small airways and the distal and peribronchiolar parenchyma. For the large airways, we measured 10 fields of the subepithelial area at a magnification of $400\times$.

Fractional areas of each compartment were determined by image analysis, using the Image-Pro Plus 4.1 for Windows software (Media Cybernetics, Silver Spring, MD, USA). Measurements of positively stained areas were performed as previously described [24]. Staining intensity was analysed by mean colour density (weighted mean per biopsy) and presented as intensity value (white=0; black=255). Detailed information is described in the online supplementary material.

Statistical analysis

Statistical analysis was performed with the SPSS 15.0 software (SPSS, Chicago, IL, USA). Data are presented as mean$\pm$SD or median (interquartile range (IQR)), depending on data distribution. To compare data between NS, NOS and COPD groups a one-way ANOVA or Kruskal–Wallis test was used, as appropriate. Bonferroni adjustments were used for multiple analysis tests. We performed a full-factorial general linear model to assess the effects of group, sex, age and centre on the fractional areas of ECM components in different lung compartments; inner, muscle and outer layer were combined in large and small airways, and peribronchial/distal parenchyma were analysed together. The results of the general linear models are shown only for ECM components that were significantly different among groups in the univariate analyses. The complete data of general linear model analysis are presented in the online supplementary material [25].

The unpaired t-test or the Mann-Whitney test was used to compare differences between smokers and ex-smokers. Fractional areas of ECM components were compared in large *versus* small airways and in peribronchial *versus* distal parenchyma using

paired t-tests. The association between morphological and clinical data was performed using Pearson's or Spearman's coefficient tests. A p-value of 0.05 was considered significant.

RESULTS

Subjects

The characteristics of the groups are presented in table 1. The COPD group was similar to the NOS group with respect to age and smoking history, but, as expected, had lower lung function than the other two groups. The mean±SD of FEV_1/FVC was 58.3±9.8% in the COPD subjects compared with 77.5±6.5% and 83.4±7.6% for the NOS and NS, respectively ($p \leqslant 0.0001$). The FEV_1/FVC ratio in the COPD patients was below the lower limit of normal (69.6±1.7) [26]. Eight of the subjects with COPD and none of the subjects in the other two groups were receiving steroids at the time of surgery.

The NS individuals were significantly younger than NOS and COPD subjects ($p \leqslant 0.007$). There were more females than males in the NS group.

Morphometry

Between 46–67 (mean 58) large airways and 137–157 small airways (mean 147) were measured depending on the protein studied. A total of 578 large airways and 1,465 small airways were measured. The mean perimeter of large airways of NS, NOS and COPD patients were 11.8±4.6 mm, 7.4±1.5 mm and 11.3±5.3 mm ($p=0.39$), respectively. For small airways the perimeter was 1.9±0.8 mm, 2.1±1.0 mm and 2.0±1.4 mm in the NS, NOS and COPD patients ($p=0.64$), respectively. The total length of peribronchial parenchyma analysed was 12.8 mm, 12.2 mm and 12.9 mm in the NS, NOS and COPD patients ($p=0.363$), respectively. For distal parenchyma the total length in NS, NOS and COPD was 17.4 mm, 15.7 mm and 17.3 mm ($p=0.084$), respectively.

Elastic fibres

The fractional area of elastic fibres was higher in NOS compared to COPD and NS groups in the inner layer ($p<0.03$), muscle layer ($p<0.0001$) and outer layer ($p<0.001$) of the large and small airways, as well as peribronchiolar ($p<0.004$) and distal parenchyma ($p<0.02$). There were no significant differences between NS and COPD (fig. 1). Data are presented in table 2.

There was a significant effect of group and centre in the large airways ($p=0.051$ and $p=0.017$, respectively) and in the small airways ($p=0.001$ and $p=0.038$, respectively) but not of age and sex. There were significant effects of group ($p=0.001$) and sex ($p=0.012$), but not of centre and age in the parenchyma (table 2S online supplementary material).

TABLE 1 Characteristics of the study groups

	Nonsmokers	Non-obstructed smokers	COPD
Subjects	16	26	26
Age yrs	52±13#	62±8	67±9
Male/female	4/12	19/7	24/2
Non-/ex-/current smoker	16/0/0	0/16/10	0/12/14
Pack-yrs		60±34	67±33
FEV_1 % pred	108.3±16.8	97.2±11.4	65.4±13.8¶
FEV_1/FVC %	83.4±7.6	77.5±6.5	58.3±9.8¶

Data are presented as n or mean±SD. COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity. #: $p \leqslant 0.007$, significant difference of nonsmoker controls compared with the other two groups; ¶: $p \leqslant 0.0001$, significant difference of COPD compared with other two groups.

Immunohistochemical analysis

Immunoreactivity of ECM proteins showed similar patterns of staining in the lung tissue of COPD patients, NOS and NS. The complete immunohistochemical data are presented in table 2.

Type-I collagen

The fractional area of type-I collagen in the inner layer of large airways and in the inner layer and muscle layer of small airways was lower in COPD patients when compared with NS ($p=0.01$, $p=0.004$ and $p=0.03$, respectively). In the outer layer of small airways, type-I collagen was lower in COPD patients and in NOS when compared to NS controls ($p \leqslant 0.01$) (fig. 2).

There were no significant effects of group, centre, age and sex on large and small airways when all layers were combined (table 3S online supplementary material).

Type-III and -IV collagen

There were no differences among COPD, NOS and NS in large or small airways and peribronchial/distal parenchyma. Results from the immunohistochemical analyses are described in table 2 and the general linear model is described in tables 4S and 5S in the online supplementary material.

Versican

Versican fractional area was lower only in the distal parenchyma of the COPD patients compared with that seen in NS ($p<0.05$) (fig. 3). There were no differences among groups for versican fractional areas in large airways, small airways and in the peribronchiolar parenchyma.

There were no significant effects of group, centre, age and sex at a parechymal level (table 6S online supplementary material).

Decorin, biglycan and lumican

There were no differences among COPD, NOS and NS in any of the large or small airway layers or peribronchial/distal parenchyma. Results from the immunohistochemical analyses are described in table 2 and the general linear model is described in tables 7S, 8S and 9S in the online supplementary material.

Fibronectin

Higher fibronectin fractional area was observed in the inner layer, muscle layer and outer layer of small airways of the COPD group compared with the NS and NOS groups ($p<0.02$, $p<0.05$ and $p<0.04$, respectively) (fig. 4). In large airways and lung parenchyma there was no difference in fibronectin fractional area among groups.

There were no significant effects of group, centre, age and sex on small airway level (table 10S online supplementary material).

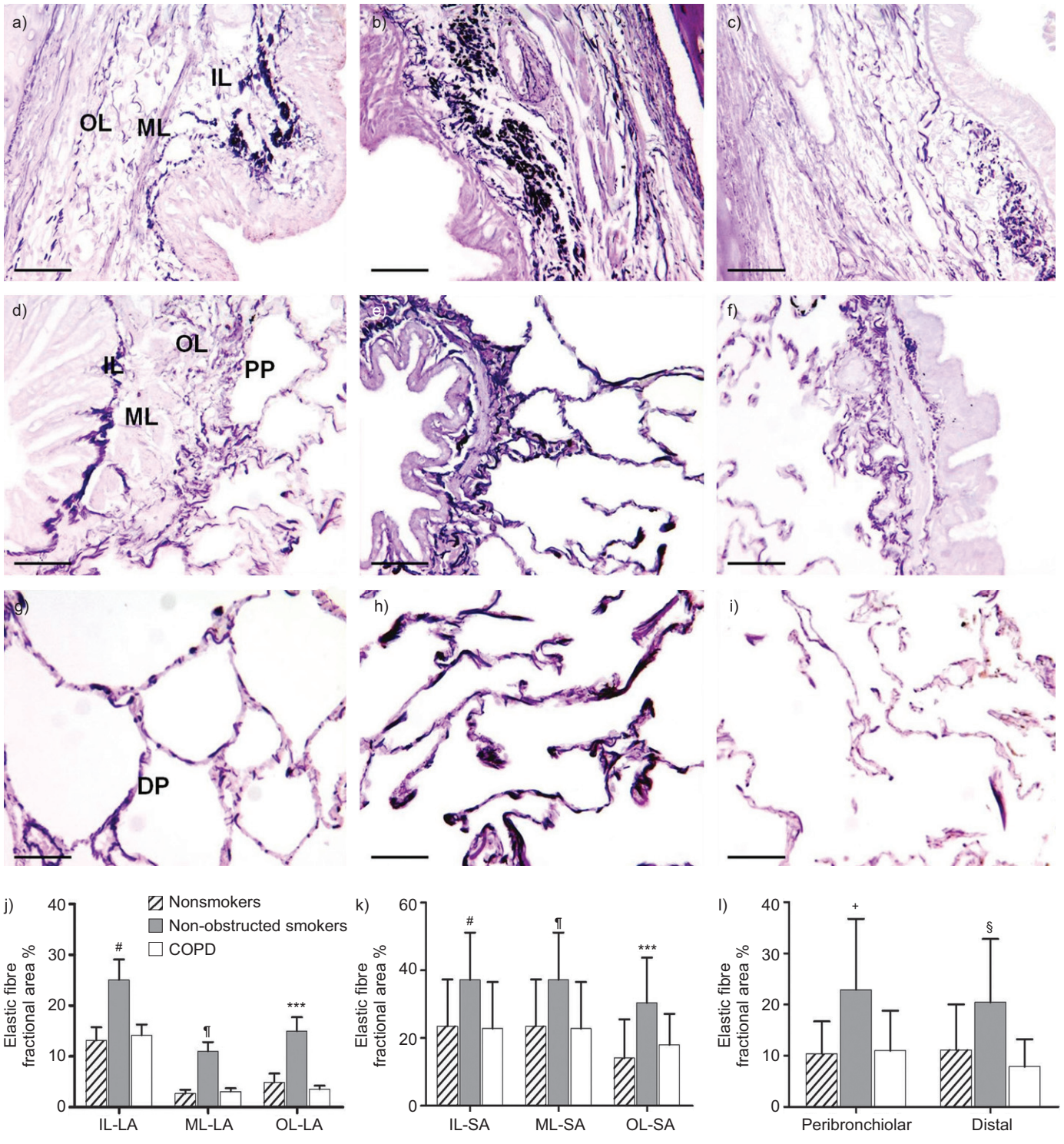

FIGURE 1. Elastic fibre fractional areas in a–c) large airways (LA), d–f) small airways (SA) and peribronchiolar parenchyma (PP), and g–i) distal parenchyma (DP) of nonsmokers (a, d and g), non-obstructed smokers (b, e and h) and chronic obstructive pulmonary disease (COPD) subjects (c, f and i). Scale bars=50 μm. j–l) Fractional areas of elastic fibres in the LA, SA, PP and DP. IL: inner layer; ML: muscle layer; OL: outer layer. Data are presented as mean±SD. ***: $p<0.001$; #: $p<0.03$; ¶: $p<0.0001$; +: $p<0.004$; §: $p<0.02$, in relation to nonsmokers and COPD subjects.

Tenascin

The fractional area of tenascin in the subepithelial area of large airways and in the inner layer of small airways was higher in the COPD group when compared with NS controls ($p<0.02$ *versus* $p<0.01$) (fig. 5). There were no differences among groups for tenascin fractional areas in muscle layer of large or small airways, or in the lung parenchyma.

TABLE 2 Fractional areas[#] of extracellular matrix components in the airways and parenchyma of nonsmokers (NS), non-obstructed smokers (NOS) and chronic obstructive pulmonary disease (COPD) patients

	Large airways			Small airways			Peribronchiolar parenchyma	Distal parenchyma	p-value[+]
	Inner layer[¶]	Muscle layer	Outer layer	Inner layer	Muscle layer	Outer layer			
Elastic fibres									
NS	13.2±7.8	2.7±2.3	4.9±5.3	23.5±13.7[ƒ]	5.1±4.1[##]	14.1±11.4[¶¶]	10.3±6.3	11.1±8.8	⩽0.042
NOS	25.1±11.4	11±5.2	15±7.8	37.2±13.9[ƒ]	18.3±12.6	30.4±13.3[¶¶]	22.9±13.9	20.5±12.4	⩽0.028
COPD	14.2±7.8	3.1±2.5	3.5±2.6	22.8±13.7	6.6±5.7[##]	18.1±9.1[¶¶]	11±7.8	7.9±5.3[++]	⩽0.025
p-value[§]	<0.03[§§]	<0.0001[§§]	<0.001[§§]	<0.03[§§]	<0.0001[§§]	<0.001[§§]	<0.004[§§]	<0.02[§§]	
Type-I collagen									
NS	14.7±11.6	9.5 (18)	19±9.8	25.4±17.7[ƒ]	8.6 (14)	34.1±15.8[¶¶]	8±4.7	6.7±4.4	⩽0.05
NOS	7.8±6.3	2 (7)	11±7.7	17.4±12[ƒ]	4.3 (10)	21.7±11.6[¶¶]	7.1±6	5.7±4.6	⩽0.027
COPD	5.3±5	1.8 (5)	12.3±10.4	11±8.6[ƒ]	3.7 (7)	17.6±9.3	5.5±3.1	4.9±3.5	0.016
p-value[§]	0.01[ƒƒ]	0.135	0.135	0.004[ƒƒ]	0.03[ƒƒ]	⩽0.01[###]	0.311	0.473	
Type-III collagen									
NS	12.3±10.8	2.6 (14)	18.7±14.5	8.6 (13)	1.9 (7)	14.4±12	0.8 (3)	1 (4)	0.85
NOS	10.9±9.1	1 (2)	11.9±13.4	8.6 (20)	2 (3)	9.9±7.6	0.5 (1)	1.7 (3)	0.91
COPD	13±15.8	0.8 (12)	13±18.3	3 (12)	1.3 (6)	10.5±12.1	0.4 (4)	1.6 (6)[++]	0.034
p-value[§]	0.904	0.678	0.547	0.294	0.710	0.407	0.474	0.782	
Type-IV collagen									
NS	1.5 (2)	31.6±20		1 (2)	26.7±14.6		24.8±12.8	22.2±11.9	0.44
NOS	2.3 (4)	27.6±15.7		1 (2)	20.6±15.4		22.1±16	28.7±15.6	0.46
COPD	2.1 (6)	22.6±13.6		2.2 (2)	19.8±15.7		26.7±13.3	20.6±10.7	0.34
p-value[§]	0.597	0.324		0.326	0.36		0.556	0.137	
Versican									
NS	25.9±16.9	7.8±8.6	9.7±6.8	38.6±22	17.6±20.3	25.1±16[¶¶]	23.3±17.5	22.2±15.5	0.004
NOS	27±20.7	14.3±21.6	21.1±17.9	36.1±21.1	13.9±12.8	26.1±16.9	17.1±14.8	13.9±13.5	0.96
COPD	38.2±18.3	22.7±20.6	20.9±14.4	48.5±16.7	21.5±14.1	34.6±18.2[¶¶]	20.8±13.9	10.9±8[++]	⩽0.026
p-value[§]	0.154	0.147	0.134	0.094	0.236	0.155	0.467	<0.05[ƒƒ]	
Decorin									
NS	11.6±13.1	7.2±7.4	19.3±14.9	15±14.1	6.6 (10)	17.9±13.4	0.7 (4)	4.4 (10)[++]	0.021
NOS	22.5±17.3	9.8±8.3	24.3±17.3	21.2±19.1	9 (21)	20.1±15.1	2.8 (7)	1.6 (3)	0.59
COPD	15.1±18.2	9±9.6	18.7±18.8	22.8±20.6	7.3 (15)	18.6±15	1 (3)	1.3 (2)	0.92
p-value[§]	0.296	0.782	0.674	0.442	0.286	0.882	0.444	0.102	
Biglycan									
NS	16.6±18.9	4.7 (14)	18.5±15.9	17.1±17.3	7.1 (12)	21.8±14.3	9.6±8.4	12.9±12.3	0.56
NOS	18.3±15.3	5.7 (8)	16.1±11.6	17.7±15.9	5.7 (11)	17.9±14.7	7±6.1	7.7±6.4	0.94
COPD	22.8±19.5	4.7 (25)	17.9±16.1	18.6±20.3	4.9 (15)	14.4±13.5	10±12.7	8.7±7.5	0.94
p-value[§]	0.659	0.693	0.918	0.965	0.76	0.304	0.590	0.220	
Lumican									
NS	20.8±18	14.9±13.3	30.7±20.4	20.8±19.2	5.2 (16)	21.8±16.4	4 (12)	13.6 (16)	0.56
NOS	31.9±27.4	16.9±15	37.5±26.8	26±21.2	7.3 (16)	21.9±17.8[¶¶]	4 (7)	6.9 (15)	0.029
COPD	26.1±19.4	21.2±17.3	30.2±20.2	31.2±22.6	10.1 (20)	25.5±16.7	4.9 (13)	6.9 (7)	0.97
p-value[§]	0.475	0.532	0.653	0.338	0.274	0.719	0.870	0.420	
Fibronectin									
NS	9.0±6.6	3.2±3.0	8.4±5.9	6.6±5.5	5.3±5.7	11.1±8.6	20±12.1	26.5±9.9	0.84
NOS	11.4±10.8	5.6±7.4	16.3±16.9	13.4±12.5	6.1±7.2	16.6±12.7	24.8±18.8	28.1±16.4	0.96
COPD	18.3±14.1	9.6±7.4	17.2±12.3	24.6±15.7	11.3±9.6	26.3±14.5[¶¶]	29.8±15.2	25.7±14.8[++]	<0.04
p-value[§]	0.136	0.074	0.284	<0.02[¶¶¶]	<0.05[¶¶¶]	<0.04[¶¶¶]	0.240	0.862	
Tenascin									
NS	24.4±25.8	6.6 (18)		0.45 (2)	15.3±14.9		3.7 (12)	12.1±8.2	0.19
NOS	44.9±31.5	4.6 (13)		13.9 (4)	8.5±9.5		2.6 (10)	11±10.7[++]	⩽0.006
COPD	56.2±23.4	8.6 (8)		25.6 (11)	17.8±16.5		4.9 (10)	10.2±7.4	0.33
p-value[§]	<0.02[ƒƒ]	0.451		<0.01[ƒƒ]	0.075		0.646	0.853	

Data are presented as mean±SD or median (interquartile range), unless otherwise stated. [#]: expressed as a percentage of the total area in each compartment; [¶]: type-IV collagen and tenascin quantification were performed in the subepithelial area of the inner layer; [+]: comparison between compartments of large airways with their respective compartment in small airways and between peribronchial and distal parenchyma (the p-value corresponds to the highest value found in the five analyses); [§]: comparison among the patient groups; [ƒ]: small airways inner layer in relation to large airways inner layer; [##]: small airway muscle layer in relation to large airways muscle layer; [¶¶]: small airways outer layer in relation to large airways outer layer; [++]: peribronchial parenchyma in relation to the distal parenchyma; [§§]: NOS in relation to NS and COPD; [ƒƒ]: COPD in relation to NS; [###]: NS in relation to NOS and COPD; [¶¶¶]: COPD in relation to NS and NOS.

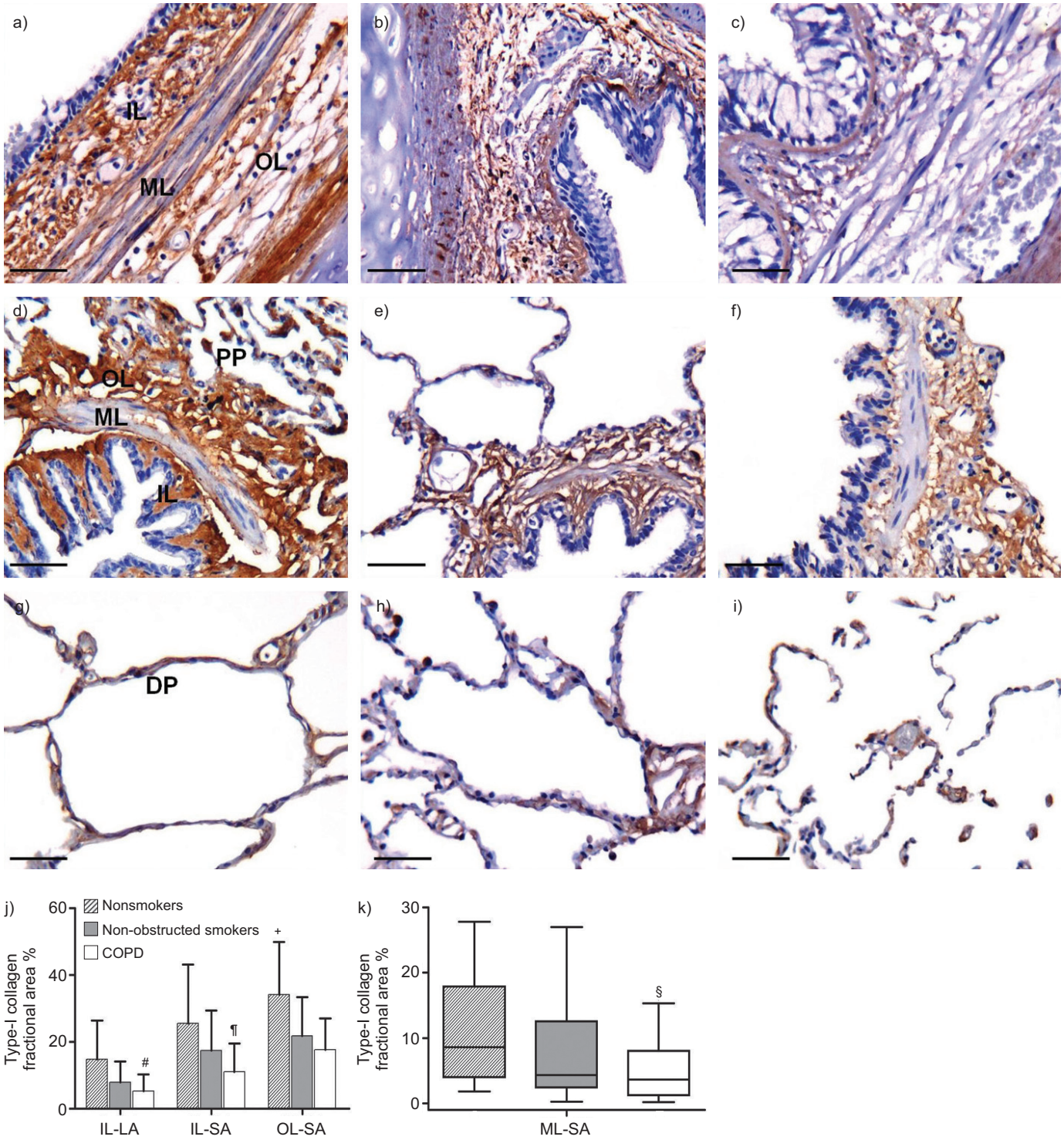

FIGURE 2. Type-I collagen fractional areas in a–c) large airways (LA), d–f) small airways (SA) and peribronchiolar parenchyma (PP), and g–i) distal parenchyma (DP) of nonsmokers (a, d and g), non-obstructed smokers (b, e and h) and the chronic obstructive pulmonary disease (COPD) subjects (c, f and i). Scale bars=50 μm. j, k) Fractional areas of type-I collagen in the LA and SA. IL: inner layer; OL: outer layer; ML: muscle layer. Data are presented as mean±SD or median (interquartile range). #: p=0.01 in relation to nonsmoker controls; ¶: p=0.004 in relation to nonsmokers; +: p⩽0.01 in relation to non-obstructed smokers and COPD subjects; §: p=0.03 in relation to nonsmokers.

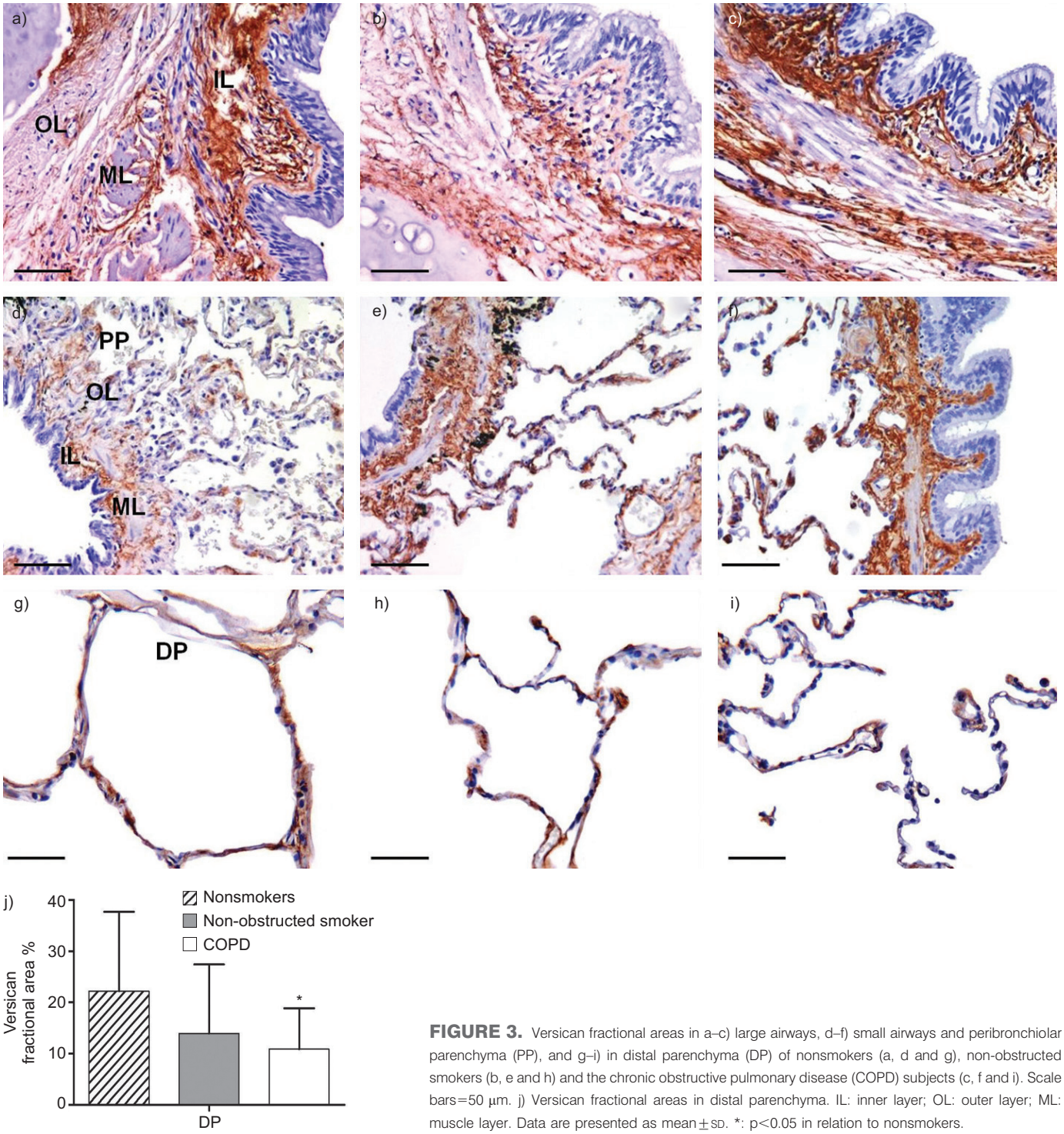

FIGURE 3. Versican fractional areas in a–c) large airways, d–f) small airways and peribronchiolar parenchyma (PP), and g–i) in distal parenchyma (DP) of nonsmokers (a, d and g), non-obstructed smokers (b, e and h) and the chronic obstructive pulmonary disease (COPD) subjects (c, f and i). Scale bars=50 μm. j) Versican fractional areas in distal parenchyma. IL: inner layer; OL: outer layer; ML: muscle layer. Data are presented as mean±SD. *: $p<0.05$ in relation to nonsmokers.

There were no significant effects of group, centre, age and sex on airways levels (table 11S online supplementary material).

Large versus *small airways/peribronchial* versus *distal parenchyma*

Differences between ECM fractional areas in large *versus* small airways and peribronchiolar and distal alveolar septa are presented in table 2.

Mean colour density

The results of mean colour density of ECM proteins were similar to those shown in the fractional area (data not shown).

Clinical–morphological correlations

Within the COPD group, inverse correlations were found between FEV1 % pred and elastic fibre fractional area of the outer layer of large airways ($r= -0.66$, $p=0.009$) and the muscle

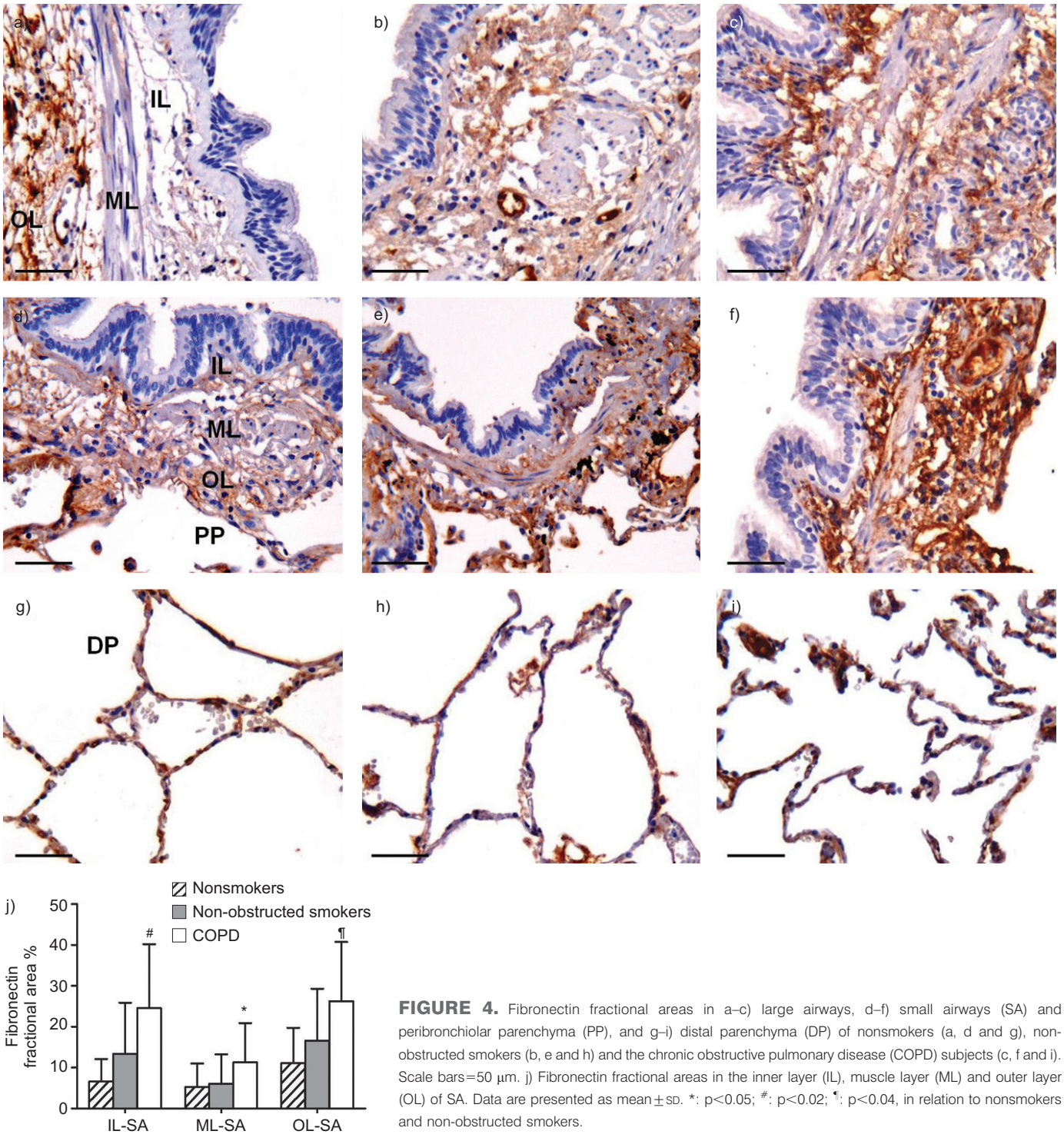

FIGURE 4. Fibronectin fractional areas in a–c) large airways, d–f) small airways (SA) and peribronchiolar parenchyma (PP), and g–i) distal parenchyma (DP) of nonsmokers (a, d and g), non-obstructed smokers (b, e and h) and the chronic obstructive pulmonary disease (COPD) subjects (c, f and i). Scale bars=50 μm. j) Fibronectin fractional areas in the inner layer (IL), muscle layer (ML) and outer layer (OL) of SA. Data are presented as mean±SD. *: $p<0.05$; #: $p<0.02$; ¶: $p<0.04$, in relation to nonsmokers and non-obstructed smokers.

layer (r= -0.48, p=0.03) of small airways; and between FEV1/FVC and fibronectin fractional area in the muscle layer of small airways (r= -0.39 p=0.05) (fig. 2S online supplementary material).

When only the NOS group was analysed, age was related to the elastic fibre fractional area of the outer layer of large airways (r=0.74, p=0.038). Inverse correlation was found between pack-yrs and elastic fibre fractional area of distal parenchyma (r= -0.59, p=0.026). Inverse correlations were also seen in fibronectin fractional areas between FEV1 % pred and the inner layer (r= -0.50, p=0.018) and outer layer (r= -0.47, p=0.027) of small airways (fig. 3S online supplementary material). There were no correlations between clinical parameters and ECM composition in the NS group.

Within COPD patients, significant correlations were seen in elastic fibre fractional areas between small airways and lung

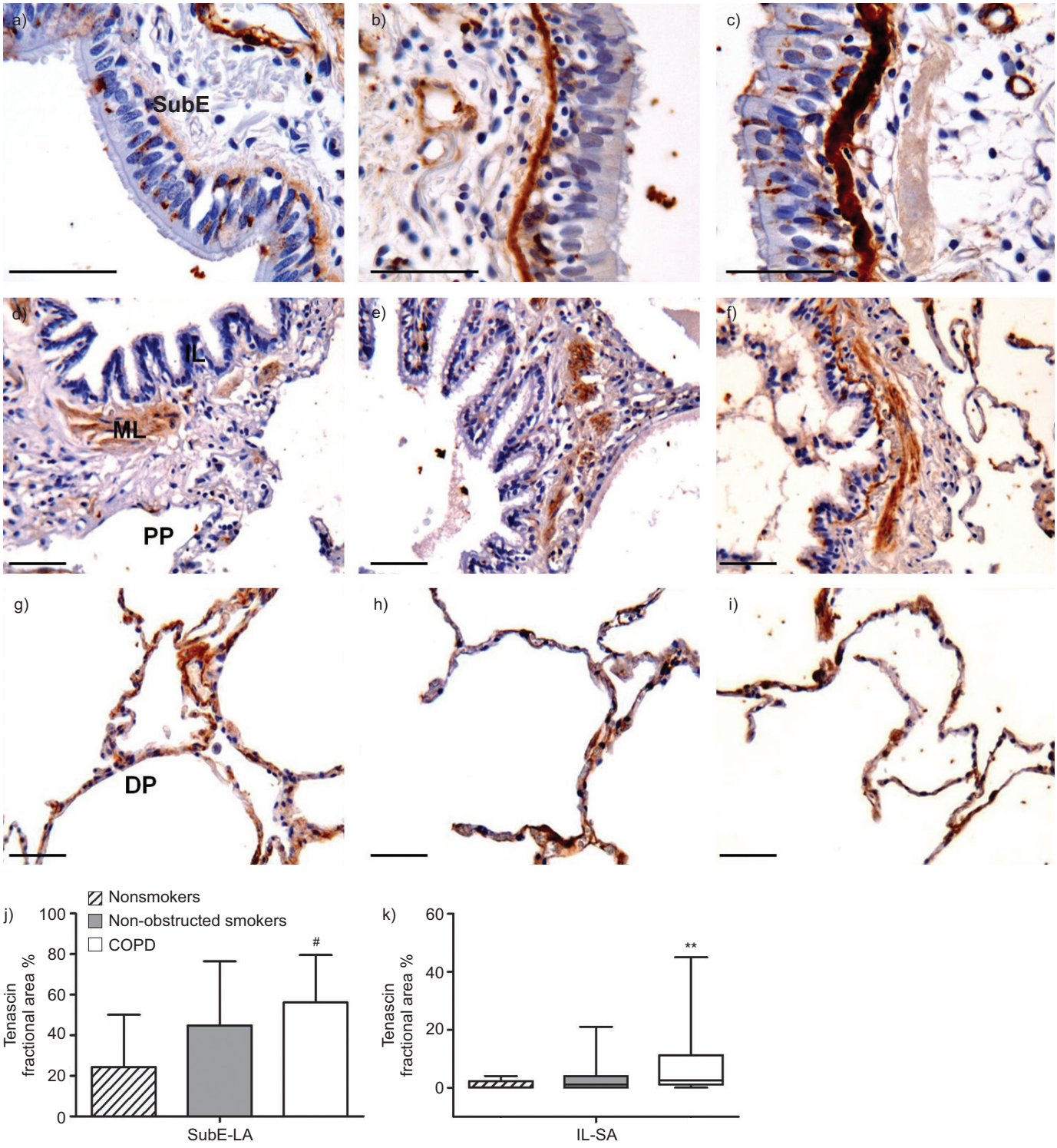

FIGURE 5. Tenascin fractional areas in a–c) large airways (LA), d–f) small airways (SA) and peribronchiolar parenchyma (PP), and g–i) distal parenchyma (DP) of nonsmokers (a, d and g), non-obstructed smokers (b, e and h) and chronic obstructive pulmonary disease (COPD) subjects (c, f and i). Scale bars=50 μm. j, k) Tenascin fractional areas in the subepithelai region (SubE), and the inner layer (IL) of (LA) and SA. ML: muscle layer. Data are presented as mean±SD. **: $p<0.01$; #: $p<0.02$, in relation to nonsmokers.

parenchyma ($r=0.77$, $p<0.0001$). Positive correlations were observed in versican fractional areas between small airways and large airways ($r=0.61$, $p<0.02$), and between small airways and lung parenchyma ($r=0.72$, $p<0.0001$). We also found significant correlations in fibronectin fractional areas between small airways and lung parenchyma ($r=0.72$, $p<0.0001$). Tenascin fractional areas in large airways correlated positively with the lung parenchyma ($r=0.61$, $p<0.02$).

Current versus *ex-smokers*

Median (IQR) duration of smoking cessation in ex-smokers was 6.0 (2.25–10) yrs. There were no significant differences in ECM composition when current smokers were compared with ex-smokers, irrespective of obstruction (data not shown).

DISCUSSION

In this study we described changes in the composition of the ECM in large and small airways and alveolar parenchyma of patients with COPD compared with smoking or nonsmoking subjects without airflow obstruction. Higher fractional areas of elastic fibres were found in NOS compared with COPD patients and NS subjects. The expression of type-I collagen in the large and small airways and of versican fractional area in distal parenchyma was lower in COPD compared with NS. The fractional areas of the fibronectin and tenascin were higher in small and large (tenascin) airways of patients with COPD. These results were not influenced by smoking status or pack-yrs. Our results indicate that COPD features complex alterations in ECM composition in both large and small airways.

Damage of elastic fibres is a classical concept in the pathophysiology of COPD, which may result from the elastase/anti-elastase imbalance caused by cigarette smoking [27]. BLACK *et al.* [14] demonstrated less elastic fibres in the distal lung of COPD patients compared with smokers. Our findings complement this study, since we demonstrated lower elastic fibre fractional area in the large and small airways and in the lung parenchyma of COPD patients compared with NOS. Unexpectedly, when COPD patients were compared with NS, no differences were found.

How can we interpret these findings? DESLEE *et al.* [28] demonstrated increased elastin gene expression in severe COPD patients without a significant increase in the elastic fibres density or in the desmosine content. These authors suggested that severe COPD patients might have a non-efficient repair of elastic fibres. Our findings are in line with these observations, and suggest that smokers without COPD may be able to increase elastin expression as a response to the smoke-related injury, whereas such an increase may not occur in COPD. Interestingly, CANTOR *et al.* [29] have shown that 3 months of exposure to cigarette in mice causes elastic fibre proliferation in the airways. Humans have an inability to adequately synthesise elastin in tissues beyond childhood [30]. We have not assessed the integrity/quality of elastic fibres in this study, but elastic fibre breakdown occurs both in NOS and COPD patients [31, 32]. Therefore, it is likely that in smokers elastic fibres may also not be fully functional.

The current inverse associations between airway elastic fibres content and FEV_1 amongst patients with COPD are in line with previous data [28]. These data suggest that airflow limitation and/or airway collapse are less common in patients with the lowest airway elastic fibres content. One may speculate that lowered elastic fibres content reduces airway wall compliance thereby increasing airway wall stiffness, which indeed has been observed in COPD [33] and even in asthma [34]. Hence, in the presence of COPD lowered airway elastic fibres content may even represent a protective mechanism against airflow limitation. Notably, in a combined analysis of COPD patients and asymptomatic smokers, airway elastic fibres content have been reported to be positively associated with spirometric values [14]. The latter was confirmed by including asymptomatic smokers in the current analysis (data not shown) and suggests that loss of airway elastic fibres in absence of COPD may also be detrimental for lung function, similar to loss of parenchymal elastic fibres as occurs in emphysema [9].

The results of collagen studies in COPD are conflicting. While some studies demonstrated increased collagen in alveoli of COPD patients [12, 35], others showed no difference between COPD and controls [11]. Few studies have focused on collagen subtypes in the airways. HOGG *et al.* [36] demonstrated a decrease in total collagen in bronchioles of severe COPD associated with a decrease in type-I/-III collagen ratio. GOSSELINK *et al.* [37] found a decreased expression of collagen type-I, α1 (COL1A1) and collagen type-III, α1 (COL3A1) genes associated with the decline in FEV_1 in lung tissue surrounding the bronchioles and in the small airways, respectively. They suggested that the thickening of small airways could not be associated to the expression of genes related to fibrogenesis.

In this study, we demonstrated less type-I collagen fractional area in COPD patients. We speculate that the loss of structural proteins leads to a reduction of stiffness of the airways, making them more susceptible to external forces applied during normal expiration, favouring collapse. In addition, we observed lower type-I collagen content in the outer layer of small airways of NOS compared with NS. It is possible that type-I collagen structural alterations at this level contribute to the airway–parenchyma uncoupling described in smokers without COPD [38].

Versican is an abundant member of the hyalectan family of the proteoglycans in the lungs [18]. We describe smaller versican fractional area in the distal parenchyma of COPD patients compared with NS. Conversely, in mild/moderate COPD patients, MERRILEES *et al.* [10] demonstrated an increased versican staining (by semiquantitative analysis) in alveoli of COPD patients compared with smoking controls. The reason for these discrepant results is not clear, but can be associated with the different methods of analyses used in both studies.

Decorin, biglycan and lumican are small proteoglycans which interact with fibrillar collagens, participating in the maintenance of the extracellular milieu [39–41]. *In vitro* studies have indicated that fibroblasts from COPD patients present abnormal production of proteoglycans and altered expression of the transforming growth factor (TGF)-β Smad pathway when exposed to cigarette smoke or different cytokines [42, 43]. In the present study, no differences were found in decorin, biglycan and lumican expression in mild/moderate COPD when compared with NOS and NS controls. VAN STRAATEN *et al.* [11] showed that decorin and biglycan staining were decreased in the peribronchiolar area in severe compared with mild emphysema patients. Later, NOORDHOEK *et al.* [44] demonstrated that decorin production by fibroblast cultures isolated from lung tissue of patients with severe emphysema is higher in a basal situation and is more significantly down-regulated after stimulation with TGF-β than the production by fibroblasts from patients with mild emphysema. Taken together with our results, alterations in proteoglycans seem to be more pronounced in the severe forms of COPD.

Tenascin and fibronectin play important morphoregulatory roles during lung development. In adult life, both proteins are

altered after tissue injury and inflammation, regulating cell adhesion, migration and differentiation [7]. Previous studies have shown that tenascin expression in large airways was altered in COPD patients and in smokers [20, 45]. Our data expand on these observations, showing higher tenascin expression in large and small airways of COPD patients. Fibronectin was also higher in COPD patients, mainly at the small airway level. Interestingly, tenascin and fibronectin induce matrix metalloproteinase expression and activity [7], contributing to the perpetuation of tissue injury. The inverse correlation of fibronectin with lung function in COPD patients reinforces these suppositions.

Our study has limitations. We lack a severe COPD group, which would have contributed to a more comprehensive description of the ECM composition in this disease. Another limitation was the younger age of the NS group, but multivariate analyses showed no significant effect of age in the data. Interestingly, an effect of centre was observed for several proteins, suggesting that ethnic/environmental factors might affect ECM composition in the lungs and contribute to different phenotypes in COPD.

Although pulmonary tissue far from the tumour was analysed, we cannot exclude that the observed changes in ECM were affected by malignancy. As all patients had malignancies, it is unlikely that an interaction would occur only in the COPD group. Some of the COPD patients did not have lung function assessed following bronchodilator; however, these older adults were or had been heavy smokers, and had no history of asthma, lung fibrosis or bronchiectasis.

Since bronchial biopsies from large airways are being used for research purposes in COPD [46], an important question is whether disease patterns are similar in the central *versus* distal lung. Similar patterns of ECM remodelling in large and small airways were observed for elastic fibres, type-I collagen and tenascin in COPD patients. However, more significant correlations in the pattern of ECM composition in COPD patients were found between small airways and parenchyma.

In summary, we showed that alterations of the major ECM elements, elastic fibres, collagens, versican, fibronectin and tenascin, are widespread in all lung compartments of mild/moderate COPD patients. The altered ECM composition in COPD is likely to contribute to the persistent tissue injury and may have a role in the airflow obstruction characteristic of this disease.

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STATEMENT OF INTEREST

Statements of interest for P.S. Hiemstra, P.J. Sterk and K.F. Rabe can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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