ONLINE SUPPLEMENTARY MATERIAL

EXTRACELLULAR MATRIX COMPOSITION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Raquel Annoni[1], Tatiana Lanças[1], Ryan Yukimatsu Tanigawa[1], Marcus de Medeiros Matsushita[2], Sandra de Morais Fernezlian[1], Andreina Bruno[3], Luiz Fernando Ferraz da Silva[1], Peter J Roughley[4], Salvatore Battaglia[5], Marisa Dolhnikoff[1], Pieter S Hiemstra[6], Peter J Sterk[7], Klaus F Rabe[8] and Thais Mauad[1].

Authors' affiliations

1. Department of Pathology, University of Sao Paulo Medical School, Sao Paulo, Brazil. 2. University of Marilia Medical School, Marilia, Brazil. 3. Institute of Biomedicine and Molecular Immunology (IBIM), Consiglio Nazionale delle Ricerche (CNR), Palermo, Italy. 4. Shriners Hospital for Children, McGill University, Montreal, Canada. 5. Dipartimento Biomedico di Medicina Interna e Specialistica (Di.Bi.MIS), University of Palermo, Palermo, Italy. 6. Department of Pulmonology, Leiden University Medical Center, Leiden, Netherlands. 7. Department of Respiratory Medicine, Academic Medical Centre, University of Amsterdam, Amsterdam, Netherlands. 8. Department of Medicine, University of Kiel, Kiel, Germany.

METHODS

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

Subjects

We analyzed lung tissue collected from 68 patients undergoing lung resection surgery for primary or metastatic lung tumours from 2001 to 2007 at our institutions.

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

Patients were classified as: 1) Non-smokers (NS, n=16): never smokers, $FEV_1 \geq 80\%$ predicted and $FEV_1/FVC \geq 70\%$; 2) Non-obstructed smokers (NOS, n=26): current and/or ex-smokers (quit≥1 month) with normal lung function ($FEV_1 \geq 80\%$ predicted and $FEV_1/FVC \geq 70\%$); and 3) COPD (n=26): current and/or ex-smokers (quitted≥1 month) with COPD ($FEV_1/FVC < 70\%$). *Post*-bronchodilator (BD) values were available in 16 of the COPD patients (5 GOLD stage I – mild, 10 GOLD stage II – moderate and 1 GOLD stage III – severe [1]), and all showed < 12% improvement compared with the pre-bronchodilator value.

Tissue Sampling and Processing

Two to four blocks of peripheral parenchyma and one or two blocks of central airways (bronchial rings) remote from the tumour were obtained in most of the 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 hours, then routinely processed and paraffin embedded. Sections 4-µm thick were cut and stained with haematoxylin and eosin (H&E) for initial analysis. We excluded cases showing: a) diffuse inflammation or fibrotic disorders; b) neoplastic tissue; and c) poststenotic pneumonia.

Histochemistry

For identification of elastic fibres, Weigert's Resorcin–Fuchsin technique with oxidation was used, as described previously [2].

Immunohistochemistry

Antigen retrieval and primary antibodies used to label the extracellular matrix components are shown in table 1. Briefly, sections were dewaxed and hydrated. A 3% H_2O_2 solution was applied for 40 min to inhibit endogenous peroxidase activity, followed by overnight incubation with the primary antibody. The streptavidin–biotin complex (LSAB; DAKO, Glostrup, Denmark) or the non-biotinylated Novolink system (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) were used as secondary antibodies. All sections were stained within one staining session using antibodies from one batch. The primary antibody

was omitted, and substitutions of the primary antibody with an isotype-matched control antibody of the same species or PBS were used as negative controls.

Table 1S. Antibodies immunohistochemical analyses

Antibody	Pretreatment	Species	Dilution	Clone	Origin
Type-I Collagen	Citrate	Goat	1:1500	Polyclonal	US Biological, Swampscott/ MA/USA
Type-III Collagen	Trypsin	Mouse	1:750	III-53	Oncogene & Calbiochem, Darmstadt/Germany
Type-IV Collagen	Citrate	Mouse	1:20	CIV-22	DakoCytomation, Glostrup/Denmark
Versican	Trypsin	Mouse	1:1000	2-b-1	Seikagaku CO, Tokyo/Japan
Biglycan	Chondroitinase	Rabbit	1:2000	Polyclonal	Prepared by Dr. PJ Roughley [3]
Decorin	Chondroitinase	Rabbit	1:100	Polyclonal	Prepared by Dr. PJ Roughley [3]
Lumican	Chondroitinase	Rabbit	1:2500	Polyclonal	Prepared by Dr. PJ Roughley [4]
Fibronectin	Citrate	Rabbit	1:6000	Polyclonal	Dako, Glostrup/Denmark
Tenascin	Pepsin	Mouse	1:400	BC-24	Sigma, Saint Louis, MO/USA

Morphological analysis

Two large (epithelial basement membrane perimeter (Pbm)>6 mm) airways, three small (Pbm≤6 mm) airways cut in transverse section and analyzed; peribronchiolar (the site of alveolar attachments) and distal alveoli (alveolar septa positioned at least one X100 field from the small airways) were analyzed for all subjects [5].

The airway walls were subdivided into the inner layer (IL), comprising the region between the epithelium and the internal smooth muscle border, the smooth muscle layer (ML), and the outer layer (OL), located between the external

smooth muscle border and the external limit of the airway, i.e. the alveolar parenchyma (figure 1S).

In large airways, type-IV collagen and tenascin stained mainly 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 analyzed only subepithelial areas in the large airways. These were defined as a region of 12 µm below the epithelium. We further analyzed 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 400x.

For each staining, fractional areas of each compartment were determined by image analysis. Measurements of positively stained areas were performed using Image-Pro® Plus 4.1 for Windows® software (Media Cybernetics, Silver Spring, MD, USA) on a computer connected to a digital camera and coupled to a light microscope (Leica DMR; Leica Microsystems GmbH, Wetzlar, Germany). We measured the area of positive staining at a magnification of 200x in 10 randomly selected fields for each layer in large airways and the entire circumference of small airways. In the alveolar tissue, measurements were performed for 10 peribronchiolar and 10 distal randomly selected alveolar septa. Cases were coded and measurements were carried out without knowledge of the clinical data.

The area of positive staining for each antibody within the marked region was determined by colour threshold. For this purpose, different sections stained with each antibody, as well as negative controls, were used to set the threshold, which was always checked by two experienced pathologists (M. Dolhnikoff and

T. Mauad). The fractional area of a given ECM protein was expressed as a percentage of the total area in each compartment [6].

To evaluate the colour intensity, in all positive structures the software also calculated the integrated optical density, a result of the mean colour density (ranging from 0 to 255 in a black/white mask transformation) of all pixels in each positive structure multiplied by its area. The sum of integrated optical density (IOD) of each object divided by the sum of the positive area lead to the balanced mean colour density indicating the intensity of colour. The colour density value was subtracted from 255 to get higher values for more intensely stained areas.

Statistical analysis

Statistical analysis was performed with the SPSS 15.0 software (SPSS, Chicago, IL, USA). Data are presented as mean±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 tests. We performed a full-factorial general linear model to assess the effects of group, gender, age and centre on the fractional areas of all ECM components in different lung compartments (inner, muscle and outer layer were combined in large and small airways and peribronchial/distal parenchyma were analyzed together).

The Student's 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. Differences at a p-value of 0.05 were considered significant.

RESULTS

The analyses of the general linear models for the different ECM components are showed in tables 2S - 11S.

Table 2S. Parameters Estimates from General Linear Models for Elastic Fibre in Large and Small Airways and in Lung Parenchyma in Non-smokers, Non-Obstructed Smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.051] NS: 0 NOS:0.099; 0.096 COPD: 0.018; 0.758	[0.017] Brz: 0 Nth: 0.105; 0.081 Ita: 0.007; 0.860	[0.304] 0.001; 0.304	[0.344] F: 0 M: -0.019; 0.627	-	0.500
Small airway (Pbm < 6mm)	[0.001] NS: 0 NOS:0.252; 0.001 COPD: -0.057; 0.597	[0.038] Brz: 0 Nth: 0.018; 0.796 Ita: 0.234; 0.037	[0.993] 0,00001; 0.993	[0.365] F: 0 M: -0.033; 0.593	-	0.344
Lung parenchyma	[0.001] NS: 0 NOS:0.192; 0.0002 COPD: -0.040; 0.517	[0.185] Brz: 0 Nth: -0.055; 0.377 Ita: 0.122; 0.158	[0.378] 0.001; 0.378	[0.012] F: 0 M: -0.067; 0.169	-	0.400

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 3S. Parameters Estimates from General Linear Models for Type-I Collagen in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.074] NS: 0 NOS:-0.035; 0.507 COPD: -0.102 0.201	[0.097] Brz: 0 Nth: 0.060; 0.442 Ita: -0.006; 0.946	[0.646] -0.001; 0.646	[0.183] F: 0 M: 0.039; 0.456	-	0.223
Small airway (Pbm < 6mm)	[0.617] NS: 0 NOS:-0.045; 0.463 COPD: 0.026; 0.820	[0.597] Brz: 0 Nth: 0.058; 0.495 Ita: -0.125; 0.294	[0.361] -0.001; 0.361	[0.954] F: 0 M: -0.003; 0.959	-	0.129
Lung parenchyma	[0.660] NS: 0 NOS:0.025; 0.158 COPD: -0.021; 0.393	[0.048] Brz: 0 Nth: -0.074; 0.031 Ita: -0.040; 0.114	[0.213] -0.001; 0.213	[0.223] F: 0 M: 0.002; 0.930	-	0.244

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 4S. Parameters Estimates from General Linear Models for Type-III Collagen in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.782] NS: 0 NOS:-0.026; 0.781 COPD: -0.119; 0.405	[0.039] Brz: 0 Nth: 0.115; 0.278 Ita: -0.041; 0.786	[0.684] 0.001; 0.684	[0.361] F: 0 M: -0.118; 0.202	-	0.118
Small airway (Pbm < 6mm)	[0.104] NS: 0 NOS:-0.089; 0.040 COPD: -0.134; 0.036	[0.112] Brz: 0 Nth: -0.117; 0.066 Ita: 0.107; 0.220	[0.664] 0.0004; 0.664	[0.067] F: 0 M: -0.102; 0.040	COPD+Nth: 0.00004 NOS+ Nth: 0.008	0.333
Lung parenchyma	[0.073] NS: 0 NOS:-0.020; 0.183 COPD: -0.023; 0.262	[0.017] Brz: 0 Nth: -0.009; 0.649 Ita: -0.020; 0.214	[0.138] -0.001; 0.138	[0.630] F: 0 M: -0.024; 0.144	NOS+M: 0.043 COPD+Ita: 0.007 COPD+Nth: 0.001	0.429

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 5S. Parameters Estimates from General Linear Models for Type-IV Collagen in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.429] NS: 0 NOS:-0.076; 0.322 COPD: 0.151; 0.199	[0.803] Brz: 0 Nth: -0.090; 0.431 Ita: -0.022; 0.856	[0.956] 0.0001; 0.956	[0.293] F: 0 M: -0.043; 0.563	-	-0.002
Small airway (Pbm < 6mm)	[0.115] NS: 0 NOS:-0.052; 0.259 COPD: -0.133; 0.159	[0.582] Brz: 0 Nth: -0.039; 0.677 Ita: 0.009; 0.921	[0.038] 0.003; 0.038	[0.553] F: 0 M: -0.025; 0.632	-	0.050
Lung parenchyma	[0.978] NS: 0 NOS:-0.001; 0.983 COPD: 0.016; 0.877	[0.054] Brz: 0 Nth: 0.106; 0.310 Ita: 0.039; 0.786	[0.137] 0.003; 0.137	[0.477] F: 0 M: 0.007; 0.928	-	-0.043

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 6S. Parameters Estimates from General Linear Models for Versican in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.630] NS: 0 NOS:-0.095; 0.468 COPD: 0.143; 0.396	[0.321] Brz: 0 Nth: 0.049; 0.766 Ita: 0.114; 0.539	[0.523] 0.002; 0.523	[0.472] F: 0 M: -0.013; 0.906	-	0.179
Small airway (Pbm < 6mm)	[0.425] NS: 0 NOS:-0.050; 0.508 COPD: -0.171; 0.123	[0.307] Brz: 0 Nth: -0.165; 0.266 Ita: 0.177; 0.245	[0.004] 0.006; 0.004	[0.099] F: 0 M: -0.036; 0.670	NOS+Nth: 0.021	0.329
Lung parenchyma	[0.084] NS: 0 NOS:-0.088; 0.131 COPD: -0.222; 0.012	[0.551] Brz: 0 Nth:-0.209; 0.018 Ita: -0.001; 0.996	[0.067] 0.003; 0.067	[0.083] F: 0 M: -0.146; 0.031	COPD+Nth: 0.012 NOS+Nth: 0.001	0.262

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 7S. Parameters Estimates from General Linear Models for Decorin in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.172] NS: 0 NOS:0.030; 0.759 COPD: 0.323; 0.032	[0.005] Brz: 0 Nth: 0.175; 0.116 Ita: 0.132; 0.382	[0.537] -0.001; 0.537	[0.090] F: 0 M: -0.041; 0.669	-	0.303
Small airway (Pbm < 6mm)	[0.202] NS: 0 NOS:-0.014; 0.838 COPD: 0.249; 0.062	[0.005] Brz: 0 Nth: 0.010; 0.920 Ita: 0.220; 0.108	[0.294] -0.002; 0.294	[0.007] F: 0 M: -0.113; 0.137	NOS+Nth: 0.023	0.264
Lung parenchyma	[0.965] NS: 0 NOS:-0.021; 0.325 COPD: 0.016; 0.678	[0.001] Brz: 0 Nth:-0.020; 0.470 Ita: 0.036; 0.377	[0.004] -0.002; 0.004	[0.168] F: 0 M: -0.023; 0.301	COPD+Nth: 0.007 NOS+Nth: 0.0001	0.473

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 8S. Parameters Estimates from General Linear Models for Biglycan in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.043] NS: 0 NOS:-0.032; 0.708 COPD: 0.198; 0.127	[0.001] Brz: 0 Nth: 0.328; 0.013 Ita: 0.082; 0.537	[0.954] -0.0001; 0.954	[0.023] F: 0 M: -0.153; 0.074	-	0.375
Small airway (Pbm < 6mm)	[0.522] NS: 0 NOS:-0.085; 0.248 COPD: -0.081; 0.588	[0.011] Brz: 0 Nth: 0.108; 0.468 Ita: 0.095; 0.533	[0.373] 0.002; 0.373	[0.408] F: 0 M: -0.078; 0.357	-	0.161
Lung parenchyma	[0.620] NS: 0 NOS: 0.017; 0.670 COPD: -0.104; 0.073	[0.145] Brz: 0 Nth:0.001; 0.983 Ita: -0.016; 0.709	[0.709] -0.0004; 0.709	[0.302] F: 0 M: -0.051; 0.259	-	0.168

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 9S. Parameters Estimates from General Linear Models for Lumican in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.242] NS: 0 NOS:-0.039; 0.752 COPD: 0.208; 0.278	[0.014] Brz: 0 Nth: 0.234; 0.106 Ita: -0.009; 0.964	[0.629] -0.001; 0.629	[0.078] F: 0 M: -0.231; 0.067	-	0.273
Small airway (Pbm < 6mm)	[0.836] NS: 0 NOS:-0.027; 0.751 COPD: -0.093; 0.453	[0.431] Brz: 0 Nth: -0.086; 0.605 Ita: 0.035; 0.736	[0.848] 0.0004; 0.848	[0.356] F: 0 M: -0.141; 0.145	-	0.115
Lung parenchyma	[0.218] NS: 0 NOS: -0.008; 0.852 COPD: -0.081; 0.214	[0.044] Brz: 0 Nth:0.047; 0.472 Ita: 0.141; 0.030	[0.928] 0.0001; 0.928	[0.161] F: 0 M: -0.098; 0.060	-	0.114

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 10S. Parameters Estimates from General Linear Models for Fibronectin in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.376] NS: 0 NOS:-0.009; 0.894 COPD: 0.116; 0.269	[0.021] Brz: 0 Nth: 0.039; 0.699 Ita: 0.129; 0.238	[0.763] 0.001; 0.763	[0.164] F: 0 M: -0.044; 0.574	-	0.305
Small airway (Pbm < 6mm)	[0.361] NS: 0 NOS:0.009; 0.881 COPD: 0.086; 0.312	[0.110] Brz: 0 Nth: 0.019; 0.871 Ita: 0.197; 0.098	[0.183] 0.002; 0.183	[0.167] F: 0 M: 0.021; 0.752	-	0.229
Lung parenchyma	[0.756] NS: 0 NOS:0.026; 0.737 COPD: -0.034; 0.757	[0.074] Brz: 0 Nth:0.093; 0.395 Ita: 0.146; 0.347	[0.213] 0.003; 0.213	[0.190] F: 0 M: -0.032; 0.700	-	0.073

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

Table 11S. Parameters Estimates from General Linear Models for Tenascin in Large and Small airways and in lung parenchyma in Non-smokers, Non-obstructed smokers and COPD Patients

Airway size	Group [Between Subject Effects, p] Parameter Estimates B coefficient; p	Center [Between Subject Effects, p] Parameter Estimates B coefficient; p	Age [Between Subject Effects, p] Parameter Estimates B coefficient; p	Gender [Between Subject Effects, p] Parameter Estimates B coefficient; p	Interactions p	r^2 for model
Large airway (Pbm > 6mm)	[0.606] NS: 0 NOS:-0.075; 0.724 COPD: 0.251; 0.431	[0.792] Brz: 0 Nth: -0.320; 0.309 Ita: 0.134; 0.477	[0.094] 0.009; 0.094	[0.384] F: 0 M: -0.108; 0.603	-	0.196
Small airway (Pbm < 6mm)	[0.101] NS: 0 NOS:-0.050; 0.321 COPD: 0.104; 0.134	[0.409] Brz: 0 Nth: -0.071; 0.445 Ita: 0.009; 0.858	[0.866] 0.0002; 0.866	[0.188] F: 0 M: -0.024; 0.651	-	0.062
Lung parenchyma	[0.246] NS: 0 NOS:-0.002; 0.967 COPD: -0.034; 0.552	[0.017] Brz: 0 Nth:-0.063; 0.277 Ita: -0.007; 0.861	[0.804] 0.0002; 0.804	[0.579] F: 0 M: 0.001; 0.974	NOS+Nth: 0.002	0.163

NS: non-smokers; NOS: non-obstructed smokers, COPD: chronic obstructive pulmonary disease; Brz; Brazil; Nth: Netherlands; Ita: Italy; F: female; M: male

REFERENCES

1 Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, van Weel C, Zielinski J. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 2007; 176: 532-555.

2 Mauad T, Xavier ACG, Saldiva PHN, Dolhnikoff M. Elastosis and fragmentation of fibres of the elastic system in fatal asthma. *Am J Resp Crit Care Med* 1999; 160: 968-75.

3 Roughley PJ, White RJ, Magny MC, Liu J, Pearce RH, Mort JS. Non-proteoglycan forms of biglycan increase with age in human articular cartilage. *Biochem J* 1993; 295: 421–6.

4 Grover J, Chen XN, Korenberg JR, Roughley PJ. The human lumican gene. Organization, chromosomal location, and expression in articular cartilage. *J Biol Chem* 1995; 270: 21942–9.

5 Mauad T, Silva LF, Santos MA, Grinberg L, Bernardi FD, Martins MA, Saldiva PH, Dolhnikoff M. Abnormal alveolar attachments with decreased elastic fibres content in distal lung in fatal asthma. *Am J Respir Crit Care Med* 2004; 170: 857-62.

6 Araujo BB, Dolhnikoff M, Silva LF, Elliot J, Lindeman JH, Ferreira DS, Mulder A, Gomes HA, Fernezlian SM, James A, Mauad T. Extracellular matrix components and regulators in the airway smooth muscle in asthma. *Eur Respir J* 2008; 32: 61-9.

LEGEND FOR ONLINE SUPPLEMENTARY FIGURE

Figure 1S. The figure shows how the measurements of fractional areas within the airways and parenchyma compartments were determined. After manually delineating the Inner (IL), Muscle (ML) and Outer Layers (OL); and Peribronchiolar (PP) and Distal Parenchyma (DP) (yellow, red, green, black and orange line, respectively) and determining their areas by image analysis, the positive staining for a given protein was determined by colour threshold. This example shows type-I collagen staining. Part A shows a small airway and B a distal parenchyma field. In large airways measurements were performed similarly as in the small airways. Scale bar=50 µm.

FIGURE 2S. Relationship between FEV_1 values (percent predicted [% pred]) and elastic fibres (A and B) fractional areas (% [FA]); and between forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) and fibronectin (C) fractional areas of COPD patients. LA: large airways; SA: small airways; ML: muscle layer; OL: outer layer. A) r=-0.66, p=0.009; B) r=-0.48, p=0.03; C) r=-0.39, p=0.05.

FIGURE 3S. Relationship between elastic fibres fractional areas (% [FA]) and age (A) and pack-years (B) of non-obstructed smoker group. The graphs C and D correspond to correlation between FEV_1 values (percent predicted [% pred]) and fibronectin fractional areas of non-obstructed smoker patients. LA: large airways; SA: small airways; DP: distal parenchyma; IL: inner layer; OL: outer

layer. A) r=0.74, p=0.038; B) r=0.59, p=0.026; C) r=-0.50, p=0.018, D) r=-0.47, p=0.027.