Age-associated Differences in the Human Lung Extracellular Matrix

Authors and affiliations

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Abstract

Introduction: Extracellular matrix (ECM) remodelling has been associated with chronic lung diseases. However, information about specific age-associated differences in lung ECM is currently limited. In this study we aimed to identify and localize age-associated ECM differences in human lung using comprehensive transcriptomic, proteomic and immunohistochemical analyses.

Methods: Our previously identified age-associated gene expression signature of the lung was re-analysed limiting it to an aging signature based on 270 control patients (37-80 years) and focused on the Matrisome core geneset using geneset enrichment analysis. To validate the age-associated transcriptomic differences on protein level, we compared the age-associated ECM genes (FDR <0.05) with a profile of age-associated proteins identified from a lung tissue proteomics dataset from 9 control patients (49-76 years) (FDR<0.05). Extensive immunohistochemical analysis was used to localize the age-associated ECM differences in lung tissues from control patients (9-82 years).

Results: Comparative analysis of transcriptomic and proteomic data identified 7 ECM proteins with higher expression with age at both gene and protein level: COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM. With immunohistochemistry we demonstrated higher protein expression with age for COL6A2 in whole tissue, parenchyma, airway wall and blood vessel, for COL14A1 in bronchial epithelium and blood vessel, and for FBLN2 and COL1A1 in lung parenchyma.

Conclusion: Our study revealed that higher age is associated with lung ECM remodelling, with specific differences occurring in defined regions within the lung. These differences may affect lung structure and physiology with aging and as such may increase susceptibility for developing chronic lung diseases.

Keywords

Aging, extracellular matrix, lung, remodelling, airway wall, blood vessel, parenchyma, bronchial epithelium, collagen

Key messages

What is already known on this topic: *summarise the state of scientific knowledge on this subject before you did your study and why this study needed to be done.*

- In animal models, it has been demonstrated that aging alters the composition of the lung ECM, with more deposition of collagen and degradation of elastin. Similar ECM differences have been observed in age-associated chronic lung diseases, including COPD; moreover, we observed in lung tissue that several ECM genes associate differently with age in COPD patients compared to non-COPD controls(1). Detailed knowledge on age-associated changes in specific ECM proteins as well as regional differences within the lung is lacking.

What this study adds: *summarise what we now know as a result of this study that we did not know before.*

- We identified 7 age-associated ECM proteins i.e. COL1A1, COL6A1, COL6A2 COL14A1, FBLN2, LTBP4 and LUM with higher transcript and protein levels in human lung tissue with age. Extensive immunohistochemical analysis revealed significant age-associated differences for 3 of these ECM proteins in specific compartments of the lung, with the most notable differences in the blood vessels and parenchyma.

How this study might affect research, practice, or policy: s*ummarise the implications of this study.*

- The identification of age-associated differences in specific human lung ECM proteins lays a new foundation for the investigation of ECM differences in age-associated chronic lung diseases. Additionally, examining the function of these age-associated ECM proteins and their cellular interactions in lung injury and repair responses may provide novel insight in mechanisms underlying chronic lung diseases.

Introduction

Aging is a natural phenomenon that affects molecular and biological processes and subsequently also the structure and function of tissues and organs including the lung, contributing in the impairment of the tissue and organ homeostasis(2). Lung aging is characterized by structural and physiological differences including larger size of alveoli, less elasticity, thickening of the small airway wall, worse lung function, and remodelling of the extracellular matrix (ECM)(3-5). Significant changes such as decreased elastin and increased collagen deposition have been observed in lung ECM from old mice(6, 7). Several features of aging have been found to be more pronounced in chronic lung diseases including chronic obstructive pulmonary disease (COPD) with a high prevalence in the elderly(3, 8).

Given its role as a provider of the architectural structure, mechanical support and regulator of several biological processes(9), the ECM plays an important role in organ homeostasis. The molecular composition, biological and mechanical properties of the ECM are tissue specific(9). The lung ECM is composed of a complex combination of elastin, collagens, glycosaminoglycans, proteoglycans and glycoproteins. Components of the ECM have specific functions in organs, accordingly elastin provides the lung its extension and recoil properties(10), collagens provide tensile strength and regulate cellular migration and adhesion, glycosaminoglycans regulate growth factor activity and lung viscoelasticity through their hydration properties(9). In our previous study, a gene set enrichment analysis (GSEA) revealed a significant negative enrichment of the ECM-receptor interaction pathway with age in COPD compared to non-COPD tissue, indicating that ECM genes change differently with increasing age in COPD patients compared to controls(1). In addition, a recent publication highlighted the positive enrichment of genes of the ECM pathway with age in human lung(11), and Burgstaller and colleagues showed age-associated changes in the expression of ECM proteins in mouse lung tissues(12). Altogether, these studies suggest age-related alterations in lung ECM proteins, however, detailed knowledge is still scarce and information on regional differences within the lung is lacking.

In this study we examined the age-associated differences in ECM proteins at both transcriptional and protein levels in human lung tissue derived from patients with normal lung function and no history of chronic lung disease. Additionally, we performed immunohistochemistry to assess age-associated ECM differences in specific lung compartments, i.e. whole lung tissue, lung parenchyma, airway wall, bronchial epithelium, and blood vessels.

Materials and methods

More detailed description of used methods is available in the online **supplementary file 1.**

Procurement of lung tissues

The transcriptomic and proteomic data were derived from lung tissues from control patients (For the numbers of patients used see analysis sections below) with normal lung function and no history of chronic lung disease. The characteristics of these patients were previously described by De Vries *et al* and Brandsma *et al*(1, 13). The lung tissues used for immunohistochemistry were derived from patients undergoing therapeutic lung resection surgery for cancer at the

University Medical Center Groningen (Groningen, The Netherlands), which were part of the HOLLAND (HistopathOLogy of Lung Aging aNd COPD) project, or the Mayo Clinic, (Rochester, Minnesota, USA). The HOLLAND project is a large immunohistochemistry study performed at the department of Pathology and Medical Biology of the UMCG aiming to identify and correlate differences in several ECM, inflammatory, epithelial and senescence markers in serial sections from the same lung in relation to chronic lung disease (i.e. COPD and IPF) and aging. The control patients from Groningen all had normal lung function, i.e. FEV1/FVC > 70%, no lung function data were available for the patients from Mayo Clinic. Patients were non-smokers, ex-smokers, or current smokers, with an age range between 9 and 82 years.

Transcriptomic analyses

We re-analysed our previously identified age-associated gene expression signature of the lung, limiting it to an aging signature based solely on the 270 non-disease control patients(1). In short, linear regression analysis adjusted for the potential confounders sex, smoking status (ex or current smokers) and technical variation using principal components explaining >1% of the variation was performed using R software. The three cohorts Groningen, Laval and Quebec were analysed separately and combined by a meta-analysis. To correct for multiple testing, the Benjamini-Hochberg false discovery rate (FDR) was applied. Next, we determined the enrichment of ECM-associated genes using Gene Set Enrichment Analysis (GSEA 4.1.0), including all genes present in the Matrisome geneset (NABA_Matrisome; M5889, Molecular Signatures Database v7.4). All Matrisome genes with a significant enrichment score were defined as our age-associated ECM genes.

Proteomic analyses

We assessed the association between age and protein expression in 9 non-disease control patients present in our previously published lung tissue proteomics dataset(13). The edge R package version 4.1.0 was used for linear regression of the data following a negative binomial distribution and using edgeR as described in Brandsma et al but using only age as factor involved in the regression analysis(13). FDR $p<0.05$ was considered significant.

Immunohistochemical staining

Immunohistochemical staining for collagen type I alpha 1 (COL1A1) COL6A1, COL6A2, COL14A1, fibulin-2 (FBLN2), latent transforming growth factor beta binding protein 4 (LTBP4) and lumican (LUM) was performed in paraffin-embedded lung tissue derived from 64 control patients (Figure 1A).

Image analyses

Different compartments of the lung including whole lung tissue, parenchyma, airway wall, bronchial epithelium, and blood vessel (both arteries and veins) were analysed separately. Specific regions were captured using Aperio ImageScope software V.12.4.3 (Leica Biosystems, Nussloch, Germany) and Adobe Photoshop software (Adobe Inc. California, United States) (Figure 1B). Fiji/ImageJ software (14) was used to quantify the mean intensity and percentage of positive stained area for each protein (Figure 1C). Data analyses were performed using R software V.4.0.0 (Boston, Massachusetts, USA) using the following formula's:

$$Area\ (\%) = \frac{Number\ of\ pixels\ positive\ for\ NovaRed}{number\ of\ pixels\ in\ total\ tissue} * 100$$

$$Mean\ intensity = 255 - \frac{Sum\ of\ intensities\ of\ pixels\ positive\ NovaRed}{Total\ number\ of\ pixels\ positive\ for\ NovaRed}$$

Statistical analysis

Linear regression and linear mixed model analyses with a random effect on intercept, correcting for sex and smoking status, were performed to determine the association between age and each protein using SPSS software V.27 (IBM Corp. in Armonk, NY, USA). P<0.05 was considered significant.

Results

Patient Characteristics

The clinical characteristics (Table S1) of the control patients included in the transcriptomics dataset included 192 ex-smokers and 78 current smokers with a normal lung function and an age range of 37-80 years. The clinical characteristics (Table S2) of the proteomics dataset included 9 ex-smoking controls with normal lung function and an age range of 49-76 years.

The clinical characteristics of the 64 control patients used for IHC staining are summarized in Table 1. 44 lung tissues were collected in Groningen and included 16 never-smokers, 18 ex-smokers and 10 current smokers. The 20 lung tissues collected in Rochester were all derived from never smokers.

Table 1: Patient characteristics

	Groningen	**Rochester**
Number, n	44	20
Age, years (range)	9-82	18-80
Male/female, n	16/28	5/15
Never-smoker, n	16	20
Ex-smoker, n	18	0
Current-smoker, n	10	0
FEV_1, mean +/- SEM	101 +/-15.46	NA
FEV_1/ FVC ratio, mean+/-SEM	0.76 +/- 0.05	NA

The FEV1 and FVC data were not available for lung tissues from Rochester and for few cases from Groningen. FEV_1: forced expiratory volume in 1 s, FVC: forced vital capacity, NA: not applicable

Gene expression signature for lung aging in non-disease controls

To determine the association between age and ECM gene expression, we first assessed the age-associated gene expression differences in the non-disease control lung tissues. In total 4201 probes corresponding to 4147 unique genes were significantly associated with age; of which 2247 probes coding for 2226 genes showed a higher and 1954 probes coding for 1939 genes a lower gene expression with higher age (full list in **supplementary file 2**). The top 10 most significantly higher and lower expressed genes are depicted in Table 2.

Table 2: The top 10 genes with higher and lower expression in relation to Age in non-disease control lung tissue.

Genes higher expressed with higher age				Genes lower expressed with higher age			
Probe number	**Gene name**	**Meta summary**	**Adjust BH**	**Probe number**	**Gene name**	**Meta summary**	**Adjust BH**
26059	COL16A1	0.012	1.08E-15	**44483**	GGCT	-0.007	4.26E-10
27901	EDA2R	0.025	1.02E-14	**27529**	KIAA1543	-0.010	2.72E-09
11226	GALNT6	0.013	2.63E-14	**43729**	ZNF518B	-0.010	4.18E-09
23264	MXRA8	0.001	1.38E-13	**26110**		-0.012	1.93E-07
26591	LOXL1	0.016	1.35E-12	**1257**		-0.006	3.48E-07
46136	NFASC	0.014	5.93E-12	**5658**	FANCE	-0.009	9.38E-07
36412		0.013	8.85E-12	**30969**	TMEM41B	-0.006	1.98E-06
18268	ITGBL1	0.022	1.41E-11	**4655**	EFNA1	-0.009	2.97E-06
17176	MMP2	0.011	2.15E-11	**44483**	GGCT	-0.012	3.41E-06
16116	F8	0.023	4.69E-11	**27529**	KIAA1543	-0.007	4.70E-06

Enrichment of Matrisome pathway among the age-associated gene signature in the lung tissue

As we were specifically interested in age-related differences in ECM gene expression, we next assessed the enrichment of the ECM genes among the ranked gene list using GSEA analysis for the Matrisome geneset consisting of 1026 ECM (-associated) genes of which 915 were present in our data.

The GSEA analysis showed a strong, significant positive enrichment of the Matrisome pathway ((enrichment score of 0.39) Figure 2) with a total of 318 core enriched ECM genes. The top 25 most significant core enriched ECM (-associated) genes are shown in Table 3 (full list in **supplementary file 3**).

Table 3: GSEA analysis revealed the association of ECM gene expression in non-disease control lung tissue with aging. *The top 25 ranked enriched ECM genes. ES; enrichment score.*

Gene symbol	Rank in gene list	Rank metric-score	Running ES	Core enrichment
COL16A1	1	9.26	0.006	yes
LOXL1	3	8.25	0.010	yes
MMP2	6	7.85	0.015	yes
FRZB	8	7.60	0.020	yes
LTBP2	12	7.39	0.024	yes
COL1A1	14	7.33	0.028	yes
DPT	19	7.10	0.032	yes
LTBP1	26	6.88	0.036	yes
SDC3	33	6.72	0.040	yes
COL1A2	35	6.62	0.044	yes
HTRA1	41	6.48	0.047	yes
AEBP1	44	6.42	0.051	yes
COL6A1	57	6.18	0.054	yes
C1QTNF7	65	6.03	0.057	yes
ECM2	74	5.84	0.060	yes

WNT10A	75	5.84	0.064	yes
COL3A1	85	5.77	0.067	yes
SPARC	86	5.76	0.070	yes
LOXL4	97	5.64	0.073	yes
ITIH3	111	5.53	0.076	yes
SPON1	114	5.502	0.079	yes
CTHRC1	118	5.45	0.082	yes
COL6A2	122	5.42	0.085	yes
PLXNB1	124	5.38	0.088	yes
SMOC2	129	5.32	0.091	yes

ECM proteins are among the top ranked age-associated proteins in lung tissue

Next, we determined the association between age and protein levels in the lung in the proteomic dataset. We identified 25 differentially expressed proteins, including 20 proteins of which levels were higher with higher age and 5 proteins of which the levels were lower with higher age (Figure 3). Among the 20 proteins with higher protein levels with higher age were several ECM proteins including COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM.

Overlap in age-associated ECM proteins in lung tissue on transcript and protein level

Next, we determined the overlap between the age-associated ECM genes identified in the transcriptomic analysis and the age-associated proteins in the proteomic analysis. We identified 7 ECM proteins that were significantly associated with age with correlation in the same direction in both datasets, i.e. higher levels with higher age. This included COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM (Figure 4A). The beta values for the age association of the 7 age-associated ECM genes from the transcriptomics analysis are shown in the forest plot. (Figure 4B). The 7 overlapping age-associated ECM proteins were among the top 8 proteins in the proteomics analysis (Figure 3).

Localization of age-associated ECM proteins in lung tissue

The immunohistochemically stained tissues were assessed for the localization of the age-associated ECM proteins in the lung (Figure 5). All ECM proteins including COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM showed positive staining in the parenchyma. The collagens COL1A1, COL6A1 and COL6A2, as well as FBLN2, showed positive staining in the submucosa and adventitia of the airway wall, and in the adventitia of the blood vessel. Additionally, FBLN2 staining was also present in the wall of small vessels. The localization of FBLN2 is suggestive of colocalization with elastin (15). Interestingly, COL14A1 showed positive staining in the airway adventitia, airway smooth muscle (ASM) layer and the apical region of the bronchial epithelium and macrophages. In the blood vessels, COL14A1 was localized in the endothelium and partly in the smooth muscle layer. LTBP4 staining was most prominent in blood vessel smooth muscle and was also present in the bronchial epithelium, ASM layer and macrophages. LUM staining was present in the bronchial epithelium, ASM layer, endothelium and blood vessel smooth muscle layer. An overview of the strenght and localization

of the positive stained age-associated ECM proteins in different compartments of the lung is summarized in Table 4.

Table 4: Overview of positive stained areas for age-associated ECM proteins including COL1A1, COL6A1 COL6A2, COL14A1, FBLN2, LTBP4 and LUM and their semi-quantitative score in the lung compartments.

ECM protein	Parenchyma	Airway wall			Bronchial epithelium	Blood vessel		
		SM	AA	ASM		BA	VSM	ED
COL1A1	+	++	++	-/+	-	++	+	-
COL6A1	++	+	++	+	-	++	+	+
COL6A2	++	++	++	+	-	++	+	++
COL14A1	+	+	+	+++	+++	+	++	+++
FBLN2	+	++	++	-	-	++	+++	-
LTBP4	+	+	+	++	+	-	++	-
LUM	+	+	+	+++	+++	-	++	++

COL1A1: collagen type I alpha 1, COL6A1: collagen type VI alpha 1, COL6A2: collagen type VI alpha 2, COL14A1: collagen type XIV alpha 1, FBLN2: fibulin-2, LTBP4: latent transforming growth factor beta binding protein 4, LUM: lumican, AA: airway adventitia, BA: blood vessel adventitia, ED: endothelium, BE: bronchial epithelium, ASM: airway smooth muscle, VSM: blood vessel smooth muscle, SM: submucosa, (-): no staining, (+): weak staining, (++): medium staining, (+++): strong staining.

Evaluation of age-associated ECM protein differences in lung tissue using immunohistochemistry

Following the localization of the 7 age-associated ECM proteins in lung tissue, we determined the age-association in whole lung tissue and in different lung regions, i.e., parenchyma, airway wall, bronchial epithelium and vessel walls with respect to percentage of positive stained tissue area as well as the mean intensitiy of the positive staining.

Age-association of ECM staining in whole lung tissue

Analysis of the whole tissue showed a significantly positive association between age and the percentage area and the mean intensity of the COL6A2 positive stained tissue (Figure 6A). For FBLN2, we also found a significantly a positive association between age and the percentage area of positive stained tissue (Figure 6A), but not for the mean intensity. For COL1A1, COL6A1, COL14A1, LTBP4 and LUM positive staining in whole lung tissue, no significant association with age was found.

Age-association of ECM staining in lung parenchyma

The analysis of the parenchymal regions showed a significantly positive association between age and the mean intensity of COL1A1, COL6A2 and FBLN2 positive stained parenchymal tissue (Figure 6B), but no association was found for the percentage area stained. No associations were found between age and COL6A1, COL14A1, LTBP4 and LUM positive stained parenchymal tissue.

Age-association of FBLN2 and COL6A2 staining in the airway wall

The airway wall was analysed for the percentage area and mean intensity of COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM positive staining. Age was significantly positively associated with the percentage area of FBLN2 and the mean intensity of COL6A2 in the airway wall. (Figure 7A). However, no age-associated difference was observed for the

percentage area of COL1A1, COL6A1 COL6A2, COL14A1, LTBP4 and LUM, and the mean intensity of COL1A1, COL6A1, COL14A1, FBLN2, LTBP4 and LUM positive staining.

Age-association of COL14A1 staining in the bronchial epithelium

COL14A1, LTBP4 and LUM showed positive staining in the bronchial epithelium and were therefore analysed for their association with age. The percentage area and mean intensity of COL14A1 were positively associated with age (Figure 7B). No age-association was observed for percentage area and mean intensity of LTBP4 and LUM positive staining in the bronchial epithelium.

Age-association of ECM staining in the blood vessel

The blood vessel wall was analysed for the percentage area and mean intensity of COL1A1, COL6A1, COL6A2, COL14A1, FBLN2 and LTBP4 positive staining. For LUM, the region from tunica media to endothelium of the blood vessel was analysed. FBLN2 was the only age-associated ECM protein that showed a positive association between its percentage area and age. The mean intensity of COL6A2, COL14A1 staining in vessel walls showed a significant positive association with age (Figure 7C). The mean intensity of COL1A1 staining in the blood vessel walls showed a negative association with increasing age. The percentage area of COL1A1, COL6A1, COL6A2, COL14A1, LTBP4 and LUM positive staining; and the mean intensity of COL6A1, FBLN2, LTBP4 and LUM positive staining were not associated with age.

Age-associated ECM differences are compartment specific

The results of the immunohistochemical analyses are summarized in Figure 8. With respect to the total area of positive ECM staining, COL6A2 was the age-associated ECM protein being higher expressed in all lung compartments including the parenchymal region, the airway wall, and the blood vessel; except in the bronchial epithelium region where it is not expressed. Compartment specific age-associated differences have been observed in the lung for COL1A1, COL14A1 and FBLN2 showing a higher expression in the parenchymal region, bronchial epithelium, and blood vessel wall, respectively. Surprisingly, COL1A1 expression was lower in the blood vessel with increasing age. The blood vessel and the parenchymal regions show the most prominent age-associated differences in their ECM composition.

Discussion

Our study describes the age-associated differences in human lung ECM using transcriptomic, proteomic and immunohistochemical analysis. Our results indicate that the human lung ECM remodels with normal aging. The Matrisome pathway, including both ECM and ECM-related proteins, was significantly and positively enriched among the age-associated gene signature in non-diseased control lung tissue. Comparing age-associated transcriptomic and proteomic differences in lung tissue, we identified 7 age-associated ECM (and ECM-associated) proteins being COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM which all showed higher levels in whole lung tissue with higher age. Subsequent immunohistochemical staining in different compartments of the lung revealed differences in the expression patterns of these ECM proteins with all proteins being expressed in the airway wall and blood vessels and 3 with clear expression in the bronchial epithelium. Age-associated differences were observed for COL6A2 in whole tissue, parenchyma, airway wall and blood vessels, for COL14A1 in bronchial epithelium and blood vessels, and for FBLN2 and COL1A1 in the parenchyma.

Our findings are in line with previous data showing higher expression of collagens and collagen-related proteins in the lung of 24-month-old mice and a higher collagen deposition observed in parenchymal regions of aged mice (16). Other studies specifically showed higher expression of COL1A1, COL6A1 and COL6A2, but a lower expression of COL14A1 with higher age in mouse lung tissue(17, 18).

In our study, COL6A2 was the only protein observed, being higher expressed throughout the different lung compartments. Surprisingly, no age-associated difference was observed for COL6A1 in any of the lung compartments where it was localized. COL6 plays an important role in cell-ECM interaction, through interaction with cell surface receptors including integrins(19). Additionally, COL6 enhances lung epithelial cell spreading and facilitates wound healing(20) and COL6 depletion in mice was linked to altered basement membrane structure and diminished cell-ECM interaction in the lung resulting in an altered pulmonary elasticity and less tolerance for physical exercises(19). These suggest the importance of COL6 in mechanical regulation of the lung, however it remains unresolved why only COL6A2 was higher expressed in our lung tissues and not COL6A1.Thus, it will be interesting to examine the role of COL6A2 in the lung and its implication of lung aging phenomenon.

Higher age is associated with higher expression of COL14A1 in the bronchial epithelium and blood vessel. The role of COL14A1 in collagen fibrillogenesis has been demonstrated, as it restraints collagen fibril diameter(21). Because it is involved in restraining the collagen fibres diameter, COL14A1 possibly involved in the modulation of collagen fibres in the blood vessel wall. COL14A1 is involved in the turnover and differentiation of epithelial cells(17), indicating a role for COL14A1 in the homeostasis of the bronchial epithelium. However, additional investigations are needed for better understanding of COL14A1 in the bronchial epithelium.

Lung elasticity is known to be less with higher age. FBLN2 serves as a bridge between fibrillin and elastin molecules and has shown a strong binding affinity to tropoelastin and COL4(22). Additionally, FBLN2 plays a role in the stabilization of the basement membrane by interacting with COL4(23). More FBLN2 in airway wall and blood vessel wall indicates a possible role in

the stabilization of their basement membranes in aged lung. FBLN2 has been identified as a positive regulator of transforming growth factor beta-1 (TGF-β1) activity(24, 25). FBLN2-KO mice displayed a decreased COL1 expression in the ischemic myocardium(26), which suggests that the increased expression of COL1A1 in the parenchyma in aged tissues maybe linked to increased expression of FBLN2. COL1 contributes to the tissue tensile strength, and its increased deposition may contribute to the lung stiffening with age, as significant increase in stiffness was observed in the parenchyma of old (41-60 year) compared to young (11-30 year) human lung tissues(27). Therefore, more stiffening of the lung with age is associated with the more deposition of FBLN2 and COL1A1. Surprisingly, COL1A1 expression was decreased in aged blood vessel wall, indicating differences in the regulation of COL1A1 expression in the blood vessel compared to the parenchyma and the importance of assessing age-associated differences in different structural compartments in the lung.

The expression of age-associated ECM proteins in different lung compartments may occur through activation of specific biological processes in certain cell types including fibroblasts, myofibroblast and ASM, which are recognized as the principal sources of ECM proteins. An increased proportion of fibroblasts was found in aged human lung compared to the proportion of epithelial cells(4, 28) and our previous work demonstrated a link between cellular senescence and ECM dysregulation in COPD lung fibroblasts. In addition, senescent ASM displayed an higher expression of ECM proteins with higher age(29). These observations suggest that the observed age-associated differences in the lung ECM may be associated with more cellular senescence in aged lung. Further research is needed to disentangle the mechanisms behind this and determine whether senescence could be driving the age-associated ECM changes in the lung.

We used a unique approach to assess age-related ECM differences on levels and in specific lung compartment including the whole tissue transcript and protein analysis followed by extensive, immunohistochemical analyses in specific lung regions. Using immunohistochemical analysis, we observed an association of more COL1A1, COL6A2, COL14A1, and FBLN2 expression with higher age, while no association was found for COL6A1, LTBP4 and LUM expression. Sensitivity and specificity of the antibodies as well as differences in the epitope region recognized by the antibodies may explain the differences observed in results obtained from immunohistochemical analysis compared to the proteomic analysis. The differences observed in percentage area/distribution and mean intensity/expression of the positive stained age-associated ECM proteins provide different information about distribution and amount of protein present within the tissues.

Our study also had some limitation, namely the relatively small number of 9 control patients used for the proteomic analysis compared to the numbers of control patients, 270 and 64, used for transcriptomic analysis and immunohistochemical analysis, respectively. Additionally, our study is cross-sectional and therefore makes it difficult to infer any causal relationship.

As previously mentioned, the aging lung is characterized by structural and physiological alterations. As the ECM regulates different biomechanical properties of tissue and organs, and comprises key proteins responsible for lung stiffness, elasticity, and recoil. In addition, the different ECM proteins play a role in binding and release of specific cytokines and chemokines.

Therefore, the age-associated ECM differences that we showed with differences in several collagens as well as FBLN2, that co-localizes within the elastic fibre, are likely important contributors to structural and physiological changes in the aging lung.

In summary, our study revealed age-associated differences in the lung ECM from histologically normal lung from patients with normal lung function and no history of chronic lung disease. Higher COL6A2 expression with higher age was present in all lung compartments except in the bronchial epithelium, where it is not expressed. Most differences were observed in the parenchyma and vessel walls. These ECM differences may affect lung structure and physiology with aging and as such help in understanding the development and progression of chronic lung diseases. Identifying the mechanisms regulating the ECM deposition in the aging lung will lay a strong foundation for the identification of potential triggers for the development of age-associated chronic lung diseases.

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Figure 1: Tissue processing, immunohistochemistry, and image analysis. (A) *Lung tissues were embedded in paraffin and cut in serial sections. The sections were then immunohistochemically stained for ECM proteins including, COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM. After the staining, the images were captured using the Hamamatsu NanoZoomer 2.0HT digital slide scanner at magnification of 40x. (**B**) The digital images were checked for their quality and once validated the whole tissue, parenchyma, airway wall, airway epithelium and blood vessel wall areas were isolated and cleaned (artifacts were removed). (**C**) For the image analysis, a macro was developed to separate the haematoxylin and NovaRed image components using the colour deconvolution plugin in ImageJ. Afterwards, a specific developed macro was run for each staining, followed by data analysis using R software. Figure was created with BioRender.com.*

Figure 1: Enrichment of Matrisome pathway among the age-associated gene signature in non-disease control lung tissue. *The vertical bar indicates the position and the heigh indicates the enrichment score of a specific gene of the Matrisome. Genes are ranked from the highest to the lowest expressed (from left to right) with increasing age. The red dotted line indicates the highest enrichment score (0.39) of the Matrisome with higher age.*

Figure 3: Heatmap of age-associated proteins in the lung of non-disease control patients*. The heat-map shows the results of the proteomic analysis of human lung tissue from control patients with normal lung function. The upper part of the map shows the significantly upregulated and the lower part the significantly downregulated proteins with age. For the statistical analysis, p-values were corrected using the Benjamini-Hochberg (FDR) method using a threshold of 0.05. FDR: fold discovery rate.*

Figure 4: Overlap in age-associated ECM proteins in the lung tissue on transcript and protein level. (A) *transcriptomic and proteomic results were examined for the overlapping of ECM gene and its encoded protein. Seven ECM proteins including COL1A1, COL6A1 COL6A2, COL14A1, FBLN2, LTBP4 and LUM were significantly higher with age on gene and protein level****. (B)*** *The beta-coefficients for age of the 7 ECM genes overlapping with encoded ECM proteins are shown in the forest plot. For the statistical analysis, p-values were corrected using the Benjamini-Hochberg (FDR) method using a threshold of 0.05. FDR: fold discovery rate. SE: standard error.*

Figure 5: Localization of age-associated ECM proteins in human lung tissues. *Following the immunohistochemical staining the age-associated ECM proteins were localized in the lung tissue. All the stained ECM proteins including COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM were located in the parenchyma. COL1A1, COL6A1 and COL6A2 were localized to the submucosa and airway adventitia and blood vessel adventitia. COL14A1 is localized to the ASM layer, bronchial epithelium, endothelium, blood vessel smooth muscle layer and macrophages. FBLN2 is also present in the submucosa and airway adventitia, smooth muscle layer of large blood vessel, and in the wall of small vessel. LUM and LTBP4 are both present in the bronchial epithelium, airway wall and blood vessel smooth muscle. Macrophages were also positive for LTBP4 staining, whereas LUM was localized to the ASM layer. Arrows indicate areas of positive staining. Scale bar 300 µm. COL1A1: collagen type I alpha 1, COL6A1: collagen type VI alpha 1, COL6A2: collagen type VI alpha 2, COL14A1: collagen type XIV alpha 1, FBLN2: fibulin-2, LTBP4: latent transforming growth factor beta binding protein 4, LUM: lumican, AA: airway adventitia, BA: blood vessel adventitia, ED: endothelium, BE:*

bronchial epithelium, ASM: airway smooth muscle, VSM: blood vessel smooth muscle, SM: submucosa, SV: small vessel, M: Macrophage, P: parenchyma.

Figure 6: Forest plot of regression estimates for age of the percentage area and mean intensity of age-associated ECM proteins in the whole lung tissue and parenchyma. (A) *Whole lung tissue sections form control patients without airflow limitation aged from 9-82 were immunohistochemically stained for the 7 age-associated ECM proteins and the positive staining was analysed using Image J software for the percentage area and mean intensity for COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM positive staining. The analysis of whole lung tissue showed a positive association of percentage area and the mean intensity of COL6A2 positive stained tissue with age. FBLN2 also showed a positive association with age in terms of total positive stained tissue, but no association of the mean intensity of the staining. The percentage area and mean intensity of COL1A1, COL6A1, COL14A1, LTBP4 and LUM positive stained tissue showed no association with age.* ***(B)*** *Parenchyma regions isolated from immunohistochemical stained whole lung tissue sections from control patients without airflow limitation aged from 9-82 were analysed using Image J software for the percentage area and mean intensity of COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM positive staining. The analysis of the parenchyma revealed the positive association of mean intensity of COL1A1, COL6A2 and FBLN2 positive stained tissue with age, but no association was found for the percentage area. Both mean intensity and percentage area of COL6A1, COL14A1, LTBP4 and LUM positive stained tissue showed no association with age. As statistical analysis, the linear regression model adjusted for sex and smoking was performed in SPSS software V.27. The total number of lung tissues used for these analyses varied between 62 and 64. COL1A1: collagen type I alpha 1, COL6A1: collagen type VI alpha 1, COL6A2: collagen type VI alpha 2, COL14A1: collagen type XIV alpha 1, FBLN2: fibulin-2, LTBP4: latent transforming growth factor beta binding protein 4, LUM: lumican. *Significant.*

Figure 7: Forest plot of regression estimates for age of the percentage area and mean intensity of age-associated ECM proteins in the airway wall, bronchial epithelium, and blood vessel. *Airway wall, bronchial epithelium and blood vessel were isolated from immunohistochemically stained whole lung tissue sections from control patients without airflow limitation aged from 9-82 and analysed using Image J software for the percentage area and mean intensity of COL1A1, COL6A1, COL6A2, COL14A1, FBLN2, LTBP4 and LUM in the airway wall and blood vessel, and COL14A1, LTBP4 and LUM in the bronchial epithelium.* ***A)*** *In the airway wall, age was positively associated with the percentage area of FBLN2 and mean intensity of COL6A2.* ***B)*** *In the bronchial epithelium, age was positively associated with the percentage area and mean intensity of COL14A1.* ***C)*** *The blood vessel showed a positive association between age and the percentage area of FBLN2 and mean intensity of COL6A2 and COL14A1. Age was negatively associated with the mean in intensity of COL1A1. A linear mixed model adjusted for sex and smoking was applied and the regression estimate and 95% confidence intervals for age were represented for each percentage area/mean intensity. The age-associated ECM proteins COL1A1, COL6A1, COL6A2 and FBLN2 were not expressed in the bronchial epithelium. COL1A1: collagen type I alpha 1, COL6A1: collagen type VI alpha 1, COL6A2: collagen type VI alpha 2, COL14A1: collagen type XIV alpha 1, FBLN2: fibulin-2, LTBP4: latent transforming growth factor beta binding protein 4, LUM: lumican, *significant.*

Figure 8: Summary of percentage area and mean intensity of the positive staining of age-associated ECM proteins in different compartments of the lung. *In the whole tissue and parenchyma region analysis COL1A1, COL6A2 and FBLN2 show age-associated differences in their percentage area (A and B) and/or mean intensity (F and G), respectively. The percentage area (C) of FBLN2 and the mean intensity (H) of COL6A2 are higher with higher age in the airway wall, while the bronchial epithelium shows a lower percentage area (D) and mean intensity (I) of COL14A1. In the blood vessel, the percentage area of FBLN2 (E) and the mean intensity of COL6A2 and COL14A1 (J) were higher with higher age, while the mean intensity of COL1A1 positive staining was lower with higher age. COL1A1: collagen type I alpha 1, COL6A1: collagen type VI alpha 1, COL6A2: collagen type VI alpha 2, COL14A1: collagen type XIV alpha 1, FBLN2: fibulin-2, LTBP4: latent transforming growth factor beta binding protein 4, LUM: lumican.*

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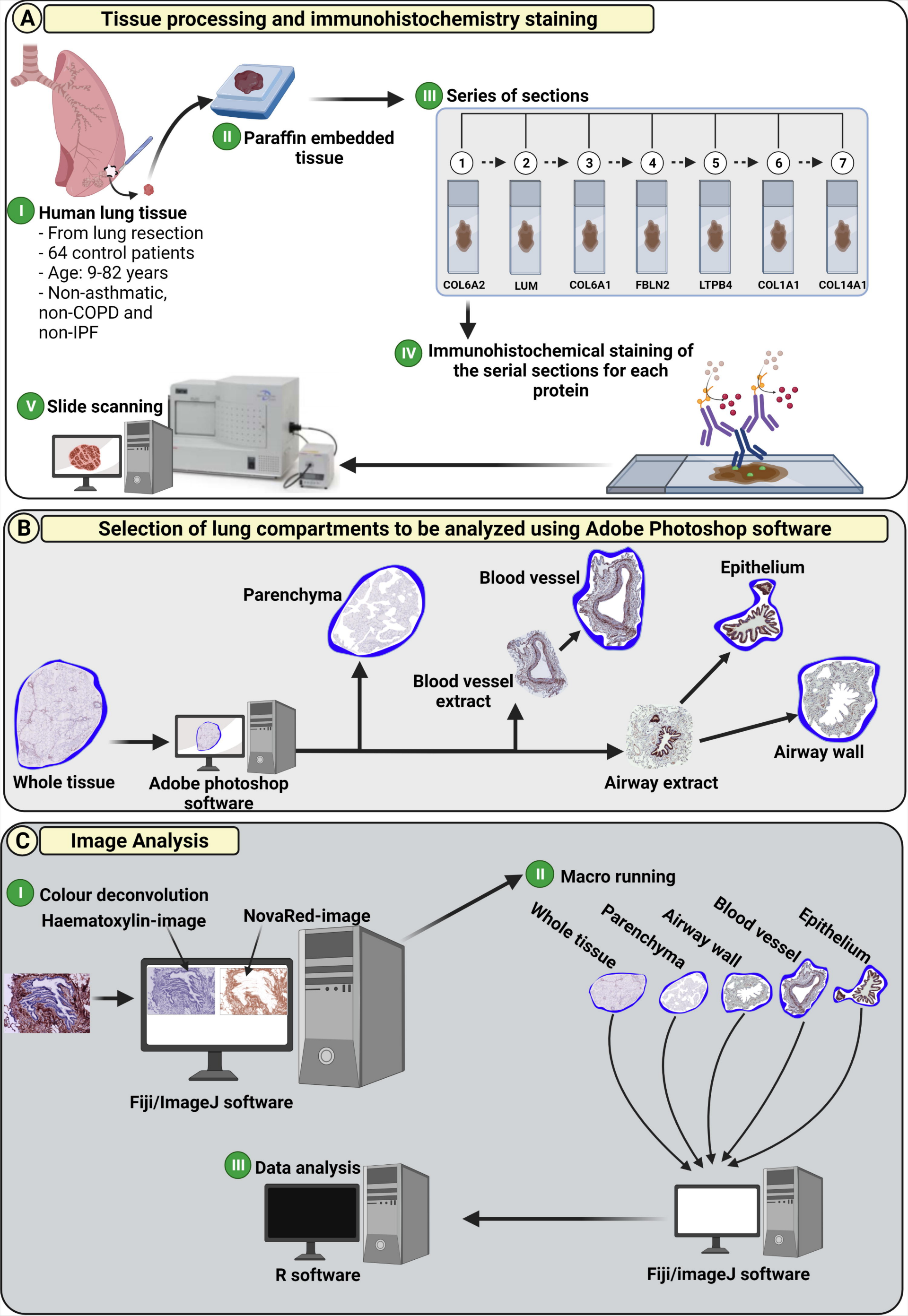
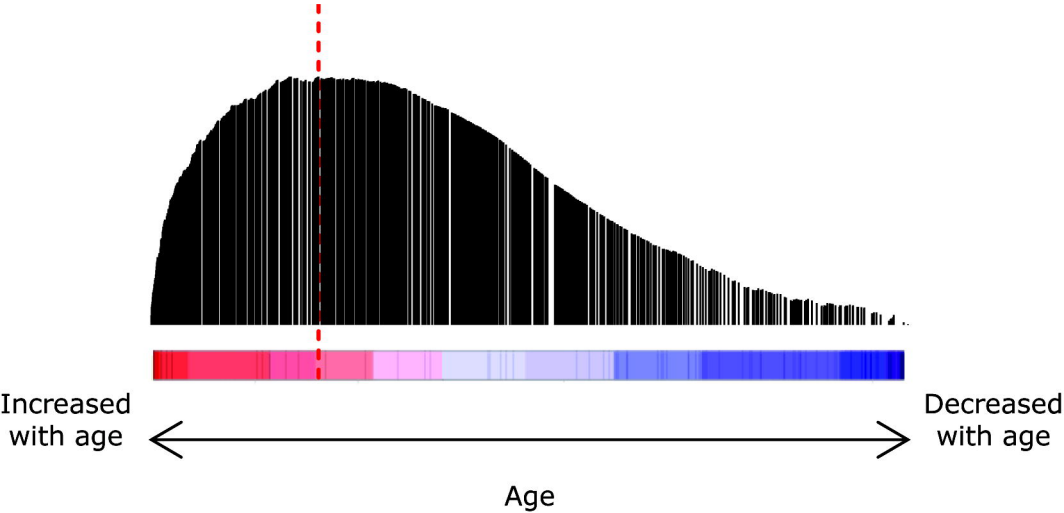
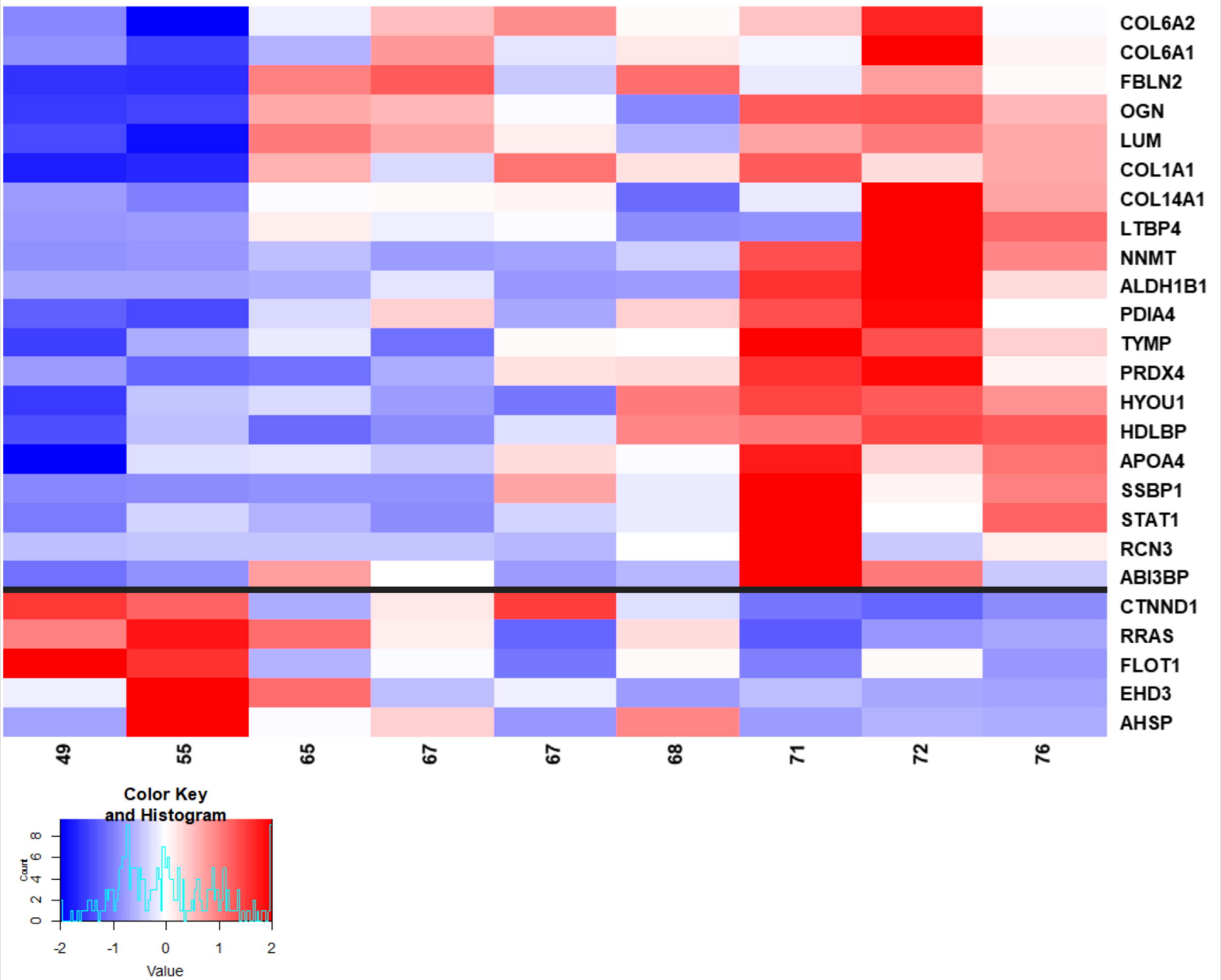
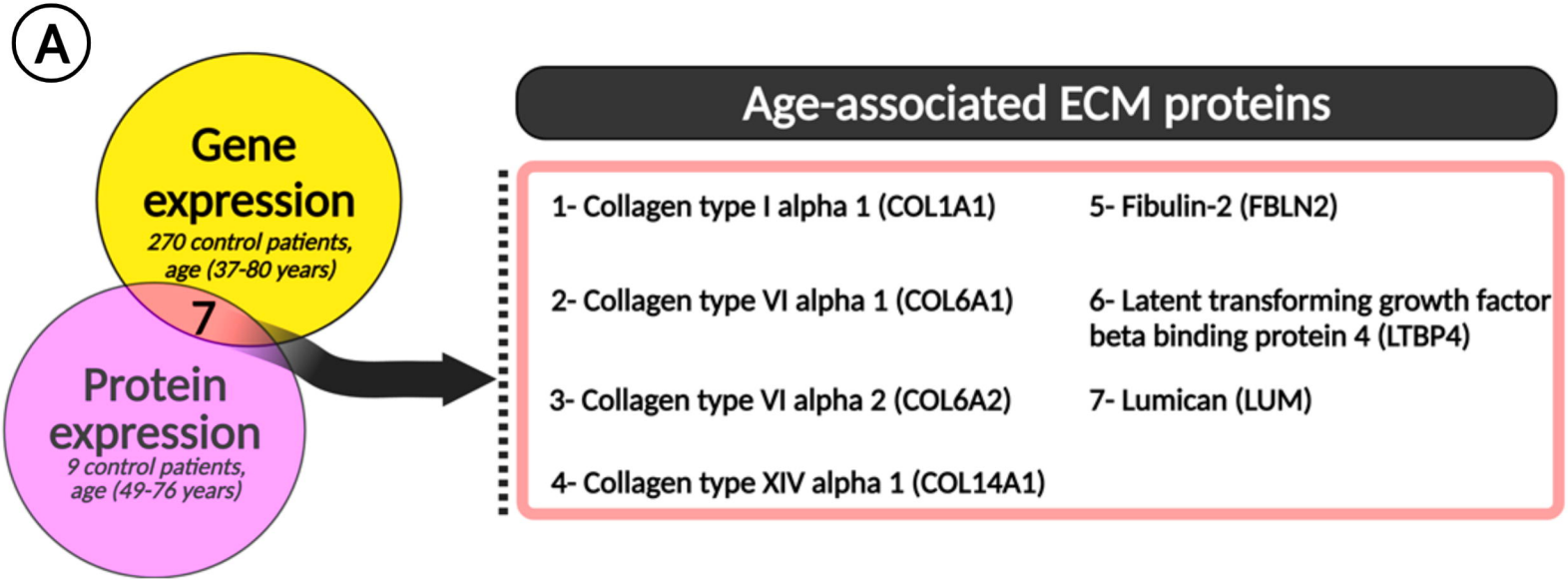

A
Tissue processing and immunohistochemistry staining
I Human lung tissue
- From lung resection
- 64 control patients
- Age: 9-82 years
- Non-asthmatic, non-COPD and non-IPF
II Paraffin embedded tissue
III Series of sections
1
2
3
4
5
6
7
COL6A2
LUM
COL6A1
FBLN2
LTPB4
COL1A1
COL14A1
IV Immunohistochemical staining of the serial sections for each protein
V Slide scanning
B
Selection of lung compartments to be analyzed using Adobe Photoshop software
Whole tissue
Adobe photoshop software
Parenchyma
Blood vessel extract
Blood vessel
Airway extract
Epithelium
Airway wall
C
Image Analysis
I Colour deconvolution
Haematoxylin-image
NovaRed-image
Fiji/ImageJ software
II Macro running
Whole tissue
Parenchyma
Airway wall
Blood vessel
Epithelium
Fiji/imageJ software
III Data analysis
R software

MATRISOME

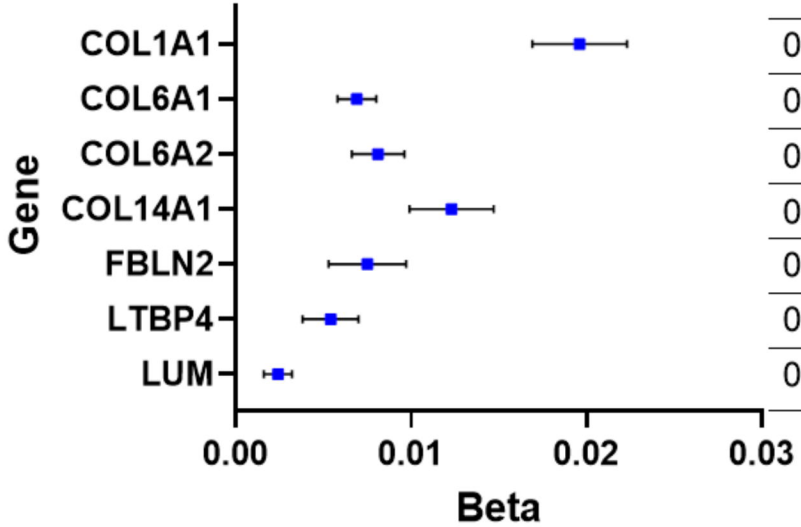



COL6A2
COL6A1
FBLN2
OGN
LUM
COL1A1
COL14A1
LTBP4
NNMT
ALDH1B1
PDIA4
TYMP
PRDX4
HYOU1
HDLBP
APOA4
SSBP1
STAT1
RCN3
ABI3BP
CTNND1
RRAS
FLOT1
EHD3
AHSP
49
55
65
67
67
68
71
72
76
Color Key
and Histogram
Count
0
2
4
6
8
-2
-1
0
1
2
Value

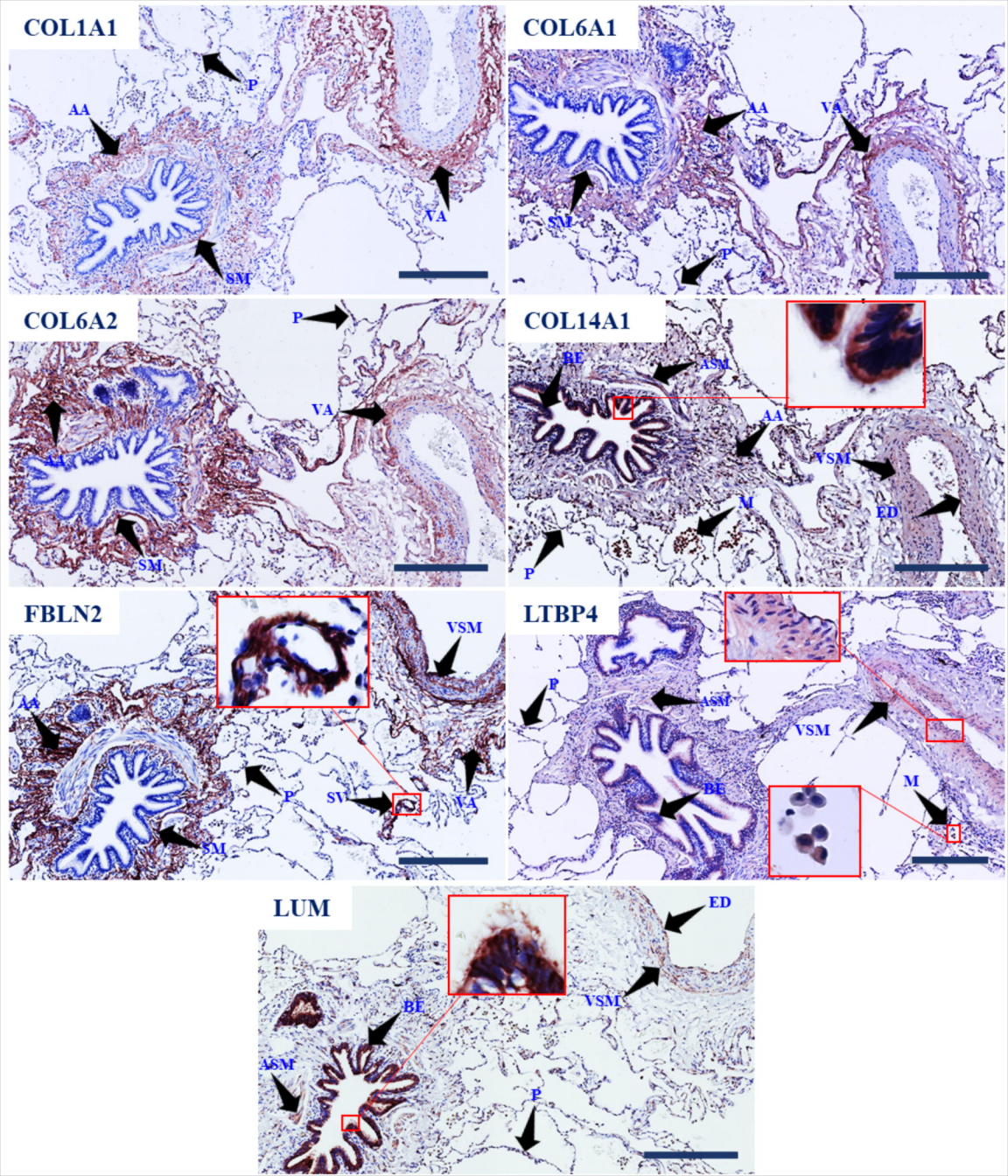


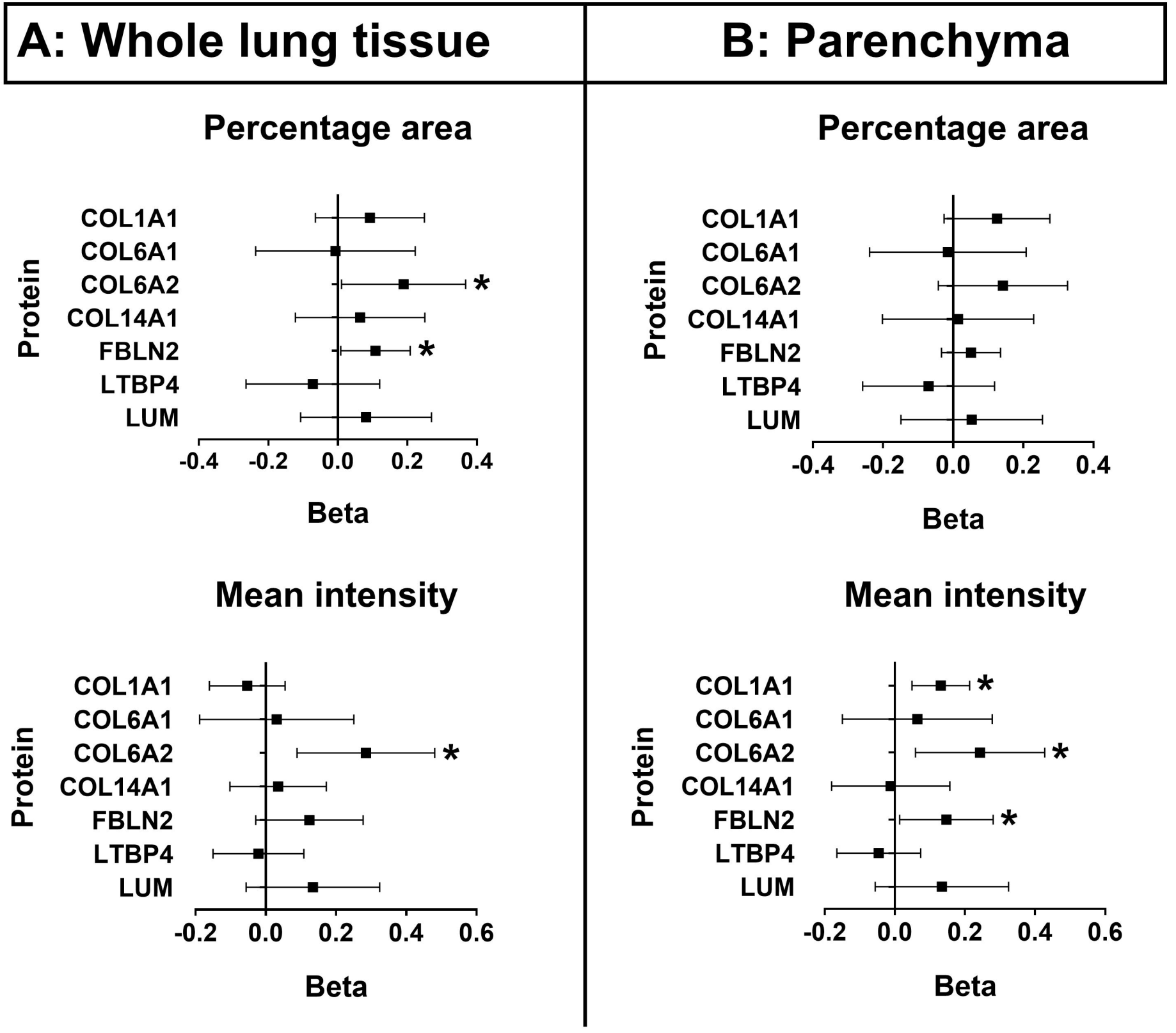
B

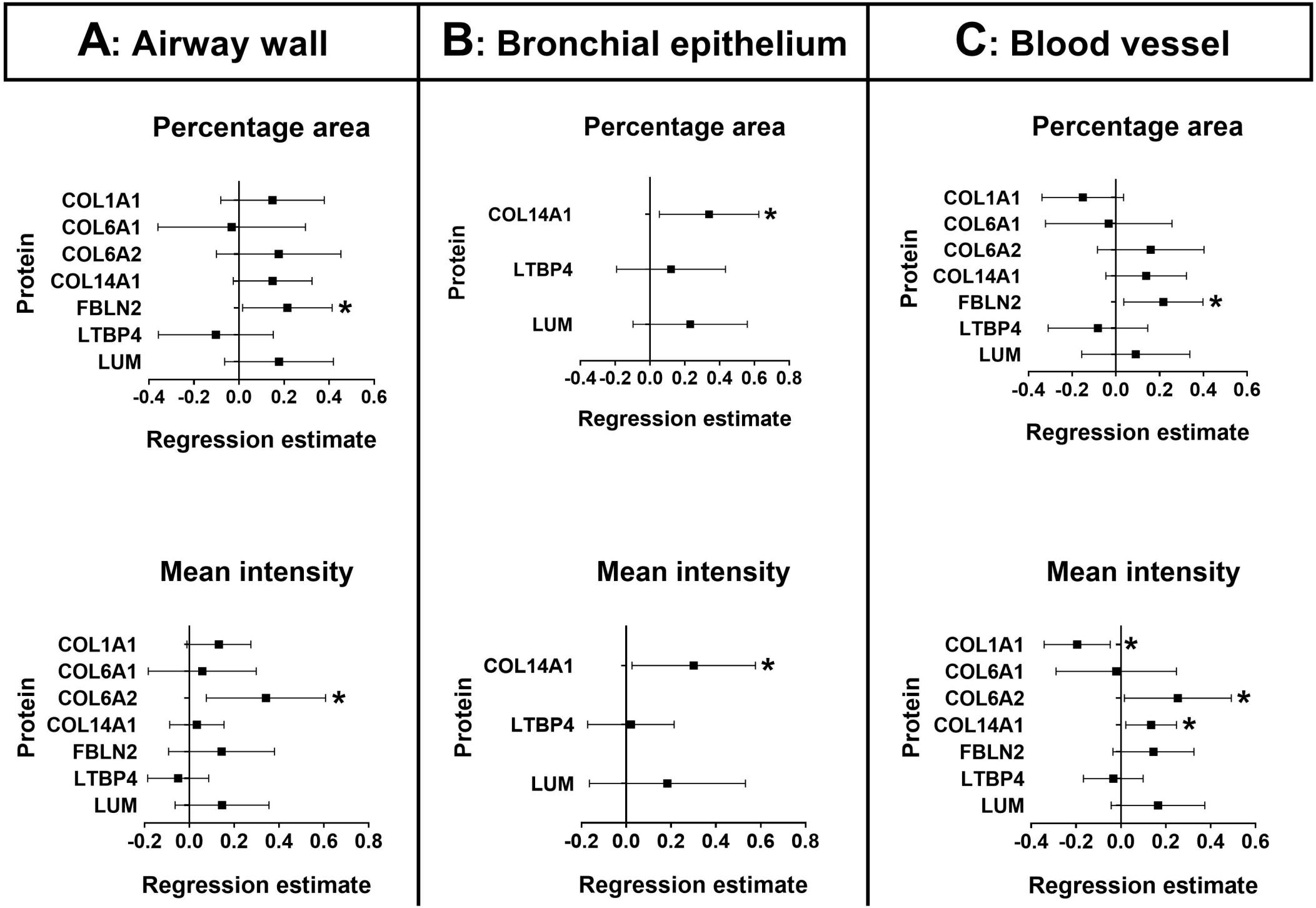
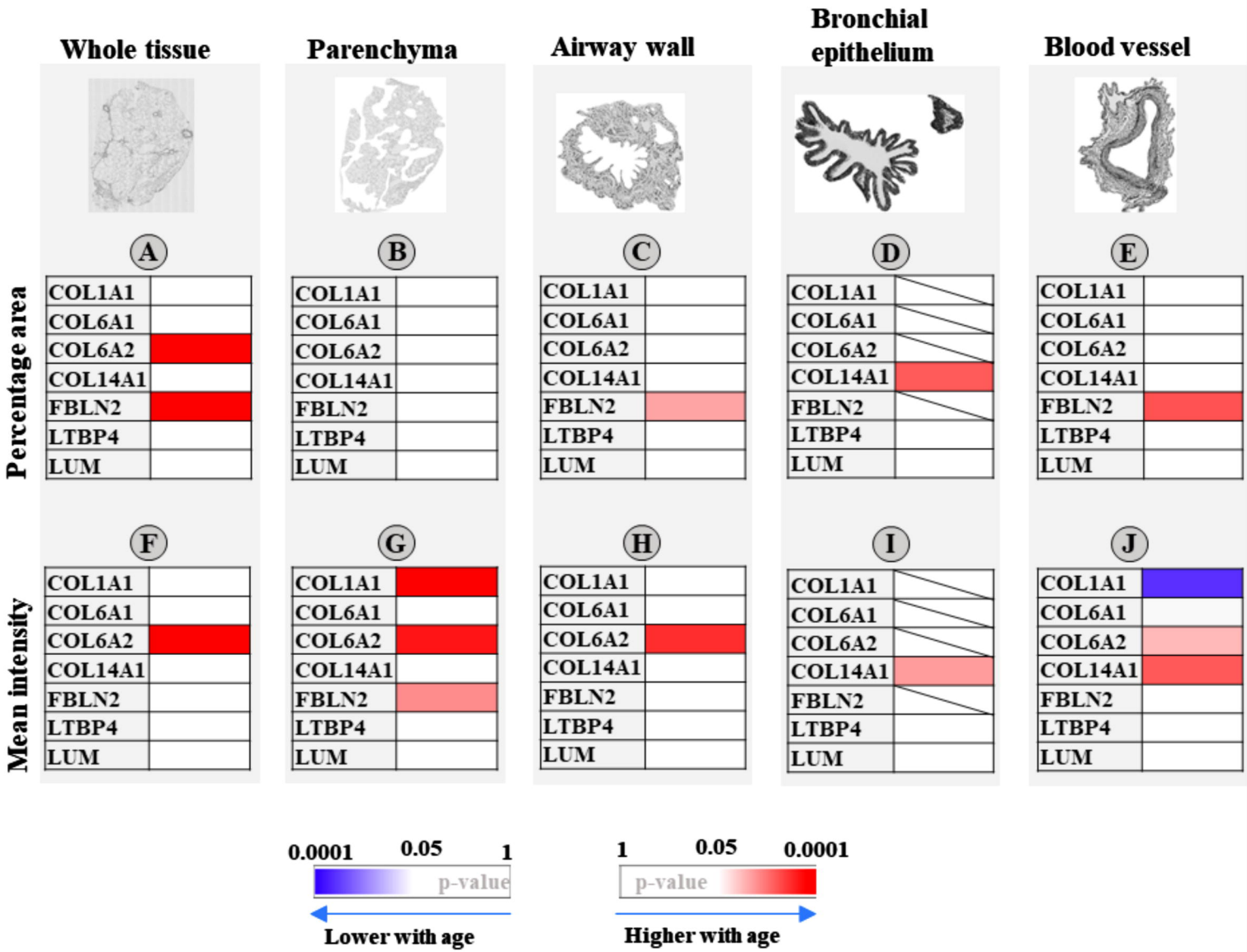
Age-associated ECM gene expression



Beta	SE	p-value
0.0196	0.0027	2.23e-13
0.0069	0.0011	6.50e-10
0.0081	0.0015	5.81e-08
0.0123	0.0024	2.84e-07
0.0075	0.0022	6.11e-04
0.0054	0.0016	7.02e-04
0.0024	0.0008	3.10e-03


COL1A1
P
AA
VA
SM
COL6A1
AA
VA
SM
P
COL6A2
P
VA
AA
SM
COL14A1
BE
ASM
AA
VSM
M
ED
P
FBLN2
VSM
AA
P
SV
VA
SM
LTBP4
P
ASM
VSM
BE
M
LUM
ED
VSM
BE
ASM
P


A: Whole lung tissue
B: Parenchyma
Percentage area
Mean intensity
Protein
COL1A1
COL6A1
COL6A2
COL14A1
FBLN2
LTBP4
LUM
*
-0.4
-0.2
0.0
0.2
0.4
0.6
Beta


A: Airway wall
B: Bronchial epithelium
C: Blood vessel
Percentage area
Mean intensity
Protein
COL1A1
COL6A1
COL6A2
COL14A1
FBLN2
LTBP4
LUM
Regression estimate
-0.4 -0.2 0.0 0.2 0.4 0.6
-0.2 0.0 0.2 0.4 0.6 0.8
-0.4 -0.2 0.0 0.2 0.4 0.6 0.8
*

Percentage area and mean intensity of age-associated ECM proteins in normal aged lung