Cellular Senescence Affects ECM Regulation in COPD Lung Tissue

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Main text word count: 3495 words

ABSTRACT

Rationale:
Higher levels of senescence have been demonstrated in COPD patients, including severe early onset (SEO)-COPD. Recently we demonstrated a link between senescence and extracellular matrix (ECM) changes in lung fibroblasts. Whether this *in vitro* observation also translates *in vivo* has not been demonstrated.

Objectives:
To determine whether senescence can contribute to COPD-associated ECM-related changes in lung tissue.

Methods:
Transcriptomics and proteomics analyses were performed on lung tissue from 60 COPD patients (including 18 SEO-COPD patients) and 32 controls. Transcript and protein levels of 471 ECM-related proteins were compared between (SEO-)COPD and control. Differentially expressed genes and proteins were correlated with six major senescence markers. Significant correlations were validated at single cell level and *in vitro*.

Results:
We identified 15 COPD- and 61 SEO-COPD-associated changes in ECM-related proteins, of which 12 and 57 at transcript and 4 and 9 at protein level, respectively. More than half (36 out of 68) of the (SEO-)COPD-associated ECM-related proteins were significantly correlated with one or more senescence markers at transcript level, with the most and strongest correlations with p21. The correlation of 6 ECM-related genes, including THBS1, ADAMTS1, and ADAMTS4, with p21 was validated at single cell level and ADAMTS1 in senescent lung fibroblasts *in vitro*.

Conclusions:
Many of the (SEO-)COPD-associated ECM-related changes in lung tissue were correlated with the senescence marker p21. As many of these ECM-related proteins are involved in ECM organization and include proteases, these results indicate a role for cellular senescence in disturbed ECM organization and protease-antiprotease imbalance in COPD.

Keywords: COPD Pathology, Emphysema, Lung Proteases, Oxidative Stress (MeSH terms)

What is already known on this topic: Accelerated ageing, including cellular senescence, has been recognized as a feature of COPD, while the functional implications of higher levels of senescence on lung tissue are largely unclear. Previously, we demonstrated a link between senescence and extracellular matrix (ECM) dysregulation in primary lung fibroblasts, but whether this translates *in vivo* has not been demonstrated yet.

What this study adds: Here, we demonstrate that cellular senescence is linked to ECM dysregulation *in vivo* in COPD lung tissue mainly by impacting the regulators of ECM organization and contributing to the protease-antiprotease imbalance in COPD.

How this study might affect research, practice or policy: Our study points towards an important functional consequence of increased senescence on disease pathology in COPD patients, including the protease-antiprotease imbalance and dysregulated elastogenesis. Our findings support the development of new therapeutic strategies of targeting senescence to restore ECM regulation in COPD.

INTRODUCTION

Accelerated ageing has been recognized to play a role in COPD pathogenesis [1, 2]. Important features of ageing that are observed in COPD lungs compared to age-matched non-COPD (ex-)smokers are lung function decline, loss of elasticity, and increased cellular senescence [3]. Cellular senescence is a major ageing hallmark demonstrated in COPD patients, both in lung tissue, and structural and immune cells [4-6]. Cellular senescence is defined by irreversible cell cycle arrest. Senescent cells have an impaired cellular function and secrete a panel of pro-inflammatory chemokines, cytokines, growth factors and extracellular matrix (ECM) proteases, called 'senescence-associated secretory phenotype' (SASP). This SASP causes chronic inflammation in the surrounding tissue and contributes to tissue dysfunction and remodelling [7, 8].

A hallmark of lung ageing is ECM dysregulation [2]. The ECM is essential for the function and structure of the lungs and is involved in healthy lung tissue repair. Lung fibroblasts are major producers of ECM and regulate ECM homeostasis. Age-related ECM changes can affect normal ECM homeostasis and healthy tissue function [9]. In COPD, fibrosis of the large and small airways is present, characterized by increased protein levels of fibronectin and collagen [10, 11]. In addition, reduced levels of proteoglycans involved in the organization of collagen crosslinking are found, including decorin and biglycan [12]. In the parenchyma of COPD patients, a reduction and breakdown of elastic fibres and other ECM proteins is observed [10, 11]. Furthermore, the protease-antiprotease balance is disturbed in COPD, which causes breakdown of the ECM mainly in the parenchyma eventually resulting in emphysema [13]. With ageing, the general ECM changes include increased fibrosis and loss of elasticity [1, 11], which is similar to what is observed in COPD, indicating a role of accelerated ageing in the dysregulated ECM in COPD patients.

Recently, we demonstrated higher levels of cellular senescence in parenchymal lung fibroblasts derived from ex-smoking COPD patients compared to matched non-COPD controls, which was most pronounced in fibroblasts from SEO-COPD patients [14]. These SEO-COPD patients are of particular interest as they have (very) severe airflow obstruction at relatively early age and therefore seem to be very susceptible to COPD development. In this previous study, we also found a link between higher levels of senescence and ECM dysregulation, including lower levels of decorin [14]. In addition, we demonstrated that induction of senescence resulted in altered expression of ECM-related genes in fibroblasts, suggesting a direct link between senescence and ECM dysregulation.

Thus, we previously demonstrated that cellular senescence leads to ECM dysregulation in parenchymal lung fibroblasts *in vitro*. The next step is to investigate whether this association is also present *in vivo* and drives ECM dysregulation in COPD patients. Therefore, we investigated whether (SEO-)COPD-associated ECM-related changes in lung tissue are associated with markers of cellular senescence using comparative transcriptomics and proteomics analyses in lung tissue from (SEO-)COPD patients and non-COPD controls. Next, significant (SEO-)COPD-associated ECM and senescence correlations in lung tissue were validated at single cell level in stromal cells using publicly available single-cell RNA sequencing data. Finally, we used our *in vitro* senescence model in lung fibroblasts for functional validation of the correlations.

MATERIALS & METHODS

The full description of the methods can be found in the online supplement.

Lung tissue collection for transcriptomic and proteomic analyses

Peripheral lung tissue from current-smoking and ex-smoking non-COPD controls (FEV_1/FVC>70% of predicted) and COPD (FEV_1/FVC<70% of predicted) patients (including SEO-COPD; FEV_1<40% of predicted and age≤55) was used from the PRoteogenomics Early onSeT cOpd (PRESTO) lung cohort (described in supplement).

Lung tissue transcriptomics

Transcriptomic data from 60 COPD patients and 32 controls was used (see table 1 for characteristics). Briefly, lung tissue cryo sections were used for RNA isolation. RNA sequencing was performed by GenomeScan B.V. A list of 471 ECM(-related) genes based on three Gene Ontology databases (GO:0085029, GO:0062023, GO:0005201) was used for differential expression analyses. ECM(-related) gene expression was compared between control current smokers (CS) and control ex-smokers (ES), between COPD and control, and between SEO-COPD and control using generalized linear models corrected for age and sex. Pearson's correlations were performed between 6 major senescence genes (*CDKN2A, CDKN1A, TP53, CDKN2B, CDKN1B,* and *LMNB1*) and the (SEO-)COPD-associated ECM(-related) genes. Multiple testing correction was done using Benjamini-Hochberg Procedure. False discovery rate (FDR) below 0.05 was considered statistically significant.

Lung tissue proteomics

From the same lung tissue cryo samples as used for transcriptomics, serial sections were used for proteomics. Briefly, discovery-based proteomics was done using a data independent acquisition method on an Orbitrap 480 LC-MS/MS platform. Protein levels of the 471 ECM(-related) proteins (as described above) were compared between control current smokers (CS) and control ex-smokers (ES), between COPD and control, and between SEO-COPD and control using generalized linear model corrected for age and sex. Pearson's correlations were performed between the 6 major senescence genes (as described above) and the (SEO-)COPD-associated ECM(-related) proteins. Multiple testing correction was done using Benjamini-Hochberg Procedure. False discovery rate (FDR) below 0.05 was considered statistically significant.

Correlation analyses in single cell dataset

To validate the significant correlations between senescence and ECM-related genes at single cell level, the publicly available single-cell RNA sequencing dataset (n=25) from the Kaminski group was used [15]. The 5 main cell populations were selected and analysed separately; stromal, epithelial, endothelial, lymphoid, and myeloid. Next, the average gene expression of each cell type per sample (Pseudobulk Mean) was calculated to avoid the influence of null counts on the correlations. CDKN1A (p21) expression was correlated with (SEO-)COPD-associated ECM(-related) genes using Spearman's correlations. For validation, a nominal p-value below 0.05 was considered statistically significant.

Senescence-induced lung fibroblast experiments

The Paraquat-induced senescence model in primary parenchymal lung fibroblasts was used as described before [14]. Senescence induction was confirmed by SA-β-gal staining, reduced proliferation, and *CDKN1A* (p21) and *LMNB1* gene expression, as described before [14]. RNA, secreted protein, and protein lysates were collected for gene expression analyses, secreted protein analyses, and Western Blot analysis.

RESULTS

Patient characteristics

The characteristics of the patients used from the PRESTO cohort for transcriptomics and proteomics analyses are shown in Table 1. In the analyses all COPD patients (FEV_1/FVC <70%pred) and the subgroup of SEO-COPD patients (FEV_1/FVC <70%pred, FEV1 <40%pred, and age ≤55) are compared to (ex-)smoker controls (FEV_1/FVC >70%pred). Smoking status was significantly different between (SEO-)COPD and control, as all SEO-COPD patients had to quit smoking before undergoing lung transplantation. In addition, age and pack-years of smoking were significantly different between SEO-COPD and control. Therefore, we corrected for age in our model.

Table 1: Patient characteristics of lung tissue subjects for transcriptomics and proteomics analyses

Variable	Non-COPD (ex-)smoker control	COPD patients	SEO-COPD patients
Number	32	60	18
Age, median (range)	62 (45-76)	59 (39-79)	52 (39-55) **#**
Male/female, N	15/17	31/29	5/13
Smoking status (ES/CS)	13/19	55/5 *****	18/0 **#**
Pack-years, median (range)	35 (10-75)	32 (6-130)	28 (6-54) **#**
non-COPD, N	32	-	-
COPD, N	-	60	18
GOLD 1	-	-	-
GOLD 2	-	14	-
GOLD 3	-	16	2
GOLD 4	-	30	16
FEV_1 %pred, median (range)	85.0 (59.8-125.0)	30.5 (10.6-77.0) *****	17.3 (12.0-37.0) **#**
FEV_1/FVC, median (range)	76.9 (71.0-86.6)	41.6 (18.8-67.9) *****	29.2 (20.5-50.0) **#**

ES = ex-smoker, CS = current smoker, FEV_1 = forced expiratory volume in the first second, FVC = Forced vital capacity, %pred = percentage of predicted. Mann–Whitney U tests or Chi-square tests were used to test differences in characteristics for COPD patients and SEO-COPD patients compared to non-COPD controls and indicated (for COPD and # for SEO-COPD) when significant (p<0.05). For transcriptomics analyses, 4 COPD samples did not pass QC and were therefore excluded for analyses, which did not affect the statistics.*

COPD-associated ECM-related genes and proteins

Of the 471 ECM(-associated) proteins selected from the Gene Ontology databases, 354 transcripts and 190 proteins were detected in the lung tissue derived transcriptomic and proteomic datasets, respectively. To avoid a possible smoking effect, we excluded differentially expressed (p<0.05) genes (n=16) and proteins (n=13) between current smoker controls and ex-smoker controls. On transcript level, 12 ECM-related genes were differentially expressed (FDR<0.05) between COPD-derived and control-derived lung tissue, with 7 genes higher expressed and 5 genes lower expressed in COPD (Figure 1A). On protein level, 4 ECM-related proteins were significantly different between COPD and control, with 3 proteins higher and 1 protein lower expressed in COPD (Figure 1B). *FBLN5* was significantly higher expressed in COPD on both transcript and protein level.

In the SEO-COPD subgroup analyses 57 ECM-related genes were differentially expressed compared to control (FDR<0.05), with 44 genes higher expressed and 13 genes lower expressed (Figure 1C). All the COPD-associated ECM-related genes were also significantly different in the same

direction in SEO-COPD. On protein level, 9 ECM-related proteins were significantly different between SEO-COPD and control, with 6 proteins higher and 3 proteins lower expressed in SEO-COPD (Figure 1D). Higher protein levels of FBLN5 and CTSH were observed for both COPD and SEO-COPD. All COPD- and SEO-COPD-associated ECM(-related) genes (57) and proteins (11) were used for further analyses.

COPD-associated ECM-related genes and proteins are correlated with markers of senescence

To assess the association between senescence and ECM, all significant (SEO-)COPD-associated ECM-related genes and proteins were correlated with 6 well-known senescence genes (*CDKN1A, CDKN1B, CDKN2A, CDKN2B, TP53*, (up with senescence) and *LMNB1* (down with senescence)). Of the 57 SEO-COPD associated ECM-related genes, 37 genes were significantly correlated with one of the senescence genes (Figure 2A). The majority of genes (26 genes) were correlated with p21, 18 with a positive and 8 with a negative correlation coefficient. All genes positively correlated with p21 were higher expressed in SEO-COPD and all negatively correlated genes were lower expressed in SEO-COPD. Most senescence-correlated ECM-related genes are regulators of ECM organization, including the three strongest correlated genes *ADAMTS4, THBS1,* and *ADAMTS1* (R = 0.84, 0.81 & 0.75 resp.) (Figure 3A-C). Some ECM-related genes were correlated with multiple senescence genes in the same direction, including *EMILIN1* and *SERPINE2*. In addition, 17 ECM-related genes were significantly correlated with *LMNB1*, with the majority (11) being positively correlated. Of the 11 (SEO-)COPD associated ECM-related proteins, 5 were positively correlated with p21, which were all higher expressed in (SEO-)COPD (Figure 2B). FBLN5 (Figure 3D) and THBS1 protein levels showed the strongest correlations with p21 (both R = 0.41), while THBS1 was also strongly correlated with p21 on transcript level (R = 0.81) (Figure 3B). The significant correlations on transcript level were used for validation at single cell level.

Validation of senescence-associated ECM-related genes at single cell level

To validate our findings in parenchymal lung tissue at single cell level we used the publicly available single cell RNA sequencing dataset from the Kaminski group [15] as this was the most representative for our lung tissue dataset, including data from parenchymal lung tissue from both COPD patients and controls. First, we checked expression levels of our ECM-related genes in the 5 major cell types and observed that the majority of ECM-related genes are predominantly expressed by stromal cells (Figure 4A), while *TGM2* and *THBS1* were predominantly expressed in endothelial and myeloid cells, respectively. As stromal cells are the main ECM producing and regulating cells, we focused on this cell population for the correlations. Of the 26 senescence-associated ECM-related genes, 6 genes correlated ($p<0.05$) with p21 in stromal cells (Figure 4B) and thus were validated at single cell level. Of these 6 genes, 4 were positively correlated and 2 were negatively correlated, which were all in the same direction as observed in lung tissue. The three strongest correlated genes in lung tissue, *ADAMTS4, THBS1,* and *ADAMTS1,* were all validated at single cell level in these stromal cells (Figure 4C). For the genes that were highest expressed in the other cell populations, we observed significant correlations with p21 for *TGM2* in endothelial and *THBS1* in myeloid cells (Supplemental Figure E1).

Functional validation in senescence-induced parenchymal lung fibroblasts

To experimentally validate the strongest correlations validated at single cell level (*ADAMTS4, THBS1, ADAMTS1)*, our senescence induction model using Paraquat in primary parenchymal lung fibroblasts was used [14]. Senescence induction was confirmed by strong induction of SA-β-gal positive cells and p21 expression and strong reduction in cell proliferation and *LMNB1* expression (Supplemental figure E2). After senescence induction the expression of *ADAMTS1* was significantly ($p<0.05$) increased as expected, while *ADAMTS4* expression was significantly decreased (Figure 5A+C). No significant differences were found on gene expression level for *THBS1* (Figure 5B). In line with gene expression, ADAMTS1 protein secretion was increased after senescence induction as well (Figure 5E). Protein secretion of THBS1 was decreased after senescence induction (Figure 5D) and secreted ADAMTS4 protein levels were below detection limit.

(Cleaved) FBLN5 changes in senescence-induced parenchymal lung fibroblasts

As FBLN5 was positively correlated with p21 on protein level and not on transcript level, we assessed FBLN5 protein levels in our senescence induction model. It has previously been described that FBLN5 protein can be proteolytically cleaved by serin proteases resulting in a 10 kDa shorter and less functional cleaved protein variant [16]. After senescence induction the protein levels of the full length FBLN5 protein (± 50 kDa) were significantly ($p<0.05$) decreased (Figure 6), whereas the absolute levels of cleaved FBLN5 (± 40 kDa) and the ratio of cleaved FBLN5 over full length FBLN5 were significantly increased after senescence induction (Figure 6B).

DISCUSSION

The aim of this study was to assess whether COPD-associated ECM-related changes are associated with cellular senescence in lung tissue and investigate whether the observed ECM dysregulation in senescent lung fibroblasts also translates *in vivo* contributing to COPD and SEO-COPD pathology. Indeed, we confirmed that many COPD-, and in particular SEO-COPD-, associated ECM-related changes are correlated with the senescence marker p21 in lung tissue. Furthermore, in an independent single cell dataset we validated the correlation with p21 for 6 of these ECM-related genes in stromal cells and for ADAMTS1 we also demonstrated a functional association between senescence induction and ADAMTS1 gene expression and protein secretion in primary lung fibroblasts. Finally, we showed that cellular senescence may play a role in the cleavage of FBLN5, resulting in a non-functional protein.

One of the important observations in our study was that among the correlations between senescence and ECM-related genes/proteins in lung tissue, there were many proteases and antiproteases, including MMP8, and several members of the ADAM (ADAM19, ADAMTS1, ADAMTS4, ADAMTS8, ADAMTS15 & ADAMTSL4) and SERPIN (SERPINB6, SERPINE2, SERPINF1 & SERPING1) protein families. This indicates that senescence affects the regulators of ECM. Subsequently, we validated the positive correlation between p21 and ADAMTS1 and ADAMTS4, which are both involved in cleavage of e.g., aggrecan and versican. Furthermore, we show that the senescence-associated ECM changes are partly driven by the stromal cell population, including fibroblasts. While the protease-antiprotease imbalance has long been recognized to play a role in the pathogenesis of COPD [17], this was mainly attributed to immune cells, including neutrophils and macrophages, whereas our results indicate involvement of stromal cell senescence, including fibroblast senescence. Interestingly, in other tissues it has already been demonstrated that senescent cells have higher expression and secretion of proteases, including MMP and ADAM proteins [8, 18, 19], supporting the hypothesis that cellular senescence may contribute to the protease-antiprotease imbalance in COPD.

ADAM/ADAMTS and SERPIN protein families are regulators of ECM homeostasis by their proteolytic and anti-proteolytic functions, respectively. Both families have important functions in inflammation, coagulation, and tissue remodelling [20]. Dysregulation of both protein families has been demonstrated in multiple pathologic inflammatory processes [13, 20]. In relation to COPD, higher gene expression of ADAMTS4 was found in COPD lung tissue [21] and higher sputum levels of ADAMTS15 were found in severe COPD patients compared to mild COPD patients [22]. The role of SERPINs in COPD has been widely described with the mutation in the SERPINA1 gene as the most described genetic risk factor for COPD, called alpha-1 antitrypsin deficiency (A1AD) [23]. In this study, we excluded this subgroup of patients. We found a correlation between several other members of the SERPIN family and senescence markers, including SERPINB6, SERPINE2, and SERPINF1, but not SERPINA1. The role of SERPINB6 and SERPINF1 in COPD has not been described yet, but genetic variants of SERPINE2 have been associated with COPD before [13] and it is described as a susceptibility gene for COPD [24]. So,

our results indicate that senescence can contribute to the dysregulation of these protein families in COPD, likely contributing to ECM dysregulation.

Multiple of the (SEO-)COPD associated ECM-related genes and proteins that were correlated with senescence have been linked to COPD before with higher gene expression or protein levels of ADAMTS4, ADAMTSL4 ADAMTS15, CLU, COL18A1, ELN, EMILIN1, FBLN5, MFAP4, MMP8, TGM2, and THBS1 [21, 22, 25-28]. Interestingly, these include glycoproteins involved in elastic fibre organization, including ELN, EMILIN1, FBLN5, MFAP4, and THBS1. We have described the role of elastogenesis genes in COPD before and proposed that these genes were higher expressed in COPD as a repair response [28]. We now show the association with senescence, a key hallmark of ageing. With ageing of tissues, including lung tissue, there is stiffening of matrix, degradation of elastic fibres and loss of elasticity [29]. In skin, higher levels of senescence in the epidermis resulted in altered elastic fibre morphology and structure [30], but what the exact role is of senescence in disturbed elastic fibre formation and structure in the lung is unclear. One of our hypotheses is that in regions with increased cellular senescence more proteases are produced (as described above) resulting in active breakdown of ECM and elastic fibres, which in turn leads to an increased need for repair and thus increased expression of elastogenesis genes.

FBLN5 has been demonstrated to be a key player in elastic fibre formation and when FBLN5 is knocked out in mice the elastic fibre formation is disturbed and these mice develop emphysema [31]. Cleavage of FBLN5 leads to a non-functional protein and *in vitro* this leads to disturbed elastogenesis [31]. While a previous study by our group demonstrated higher FBLN5 protein levels in COPD lung tissue, the level of non-functional cleaved FBLN5 seemed to be higher in COPD lung tissue as well [28]. Here, we demonstrate that senescent lung fibroblasts can contribute to these higher levels non-functional cleaved FBLN5 in COPD lung tissue. Till now, the role of non-functional cleaved FBLN5 was only shown in aged mouse skin [16], but not in relation to senescence yet.

Multiple of the (SEO-)COPD-associated ECM-related genes and proteins that correlated with senescence markers have been linked to ageing and/or age-related disease before. In addition, several have been linked to senescence before, including ADAM and SERPIN protein families, ANXA6, CLU, FBLN5, MMP8, PCOLCE, SRPX2, and THBS1 [18, 19, 32-35]. Proteases and SERPINs have been shown to be SASP proteins in multiple studies and multiples cell types, including fibroblasts and epithelial cells [8, 18, 19]. THBS1 is involved in mediating cellular senescence by promoting the cell cycle arrest and is secreted by senescent human peritoneal mesothelial cells and thus part of the SASP [36]. The role of THBS1 in COPD is not fully understood yet, but THBS1 has been demonstrated to activate TGF-β and thus may have a role in tissue remodelling in COPD [37], likely through senescence as our results suggest. The positive correlation between THBS1 and senescence was also validated in the stromal and myeloid cells at single cell level, but we could not functionally validate this using our senescence model in lung fibroblasts, indicating that likely senescence in other stromal or myeloid cells may play a role.

Most observed correlations between (SEO-)COPD-associated ECM-related genes and senescence markers were with p21. As the senescence marker p21 is mainly involved in cellular senescence induced by oxidative stress and DNA damage [7], and oxidative stress has been proposed to be a main contributor to senescence in COPD [38, 39], the strong signal for p21 in COPD is not surprising.

A limitation of our study in lung tissue, was that the correlations between senescence and ECM-related genes/proteins were based on a mix of different cell types. To get more insight into this association in stromal cells, we validated our findings on transcript level at single cell level in stromal cells derived from a different, but comparable lung tissue cohort [15]. In our paraquat-induced senescence model in lung fibroblasts, we could not validate the association with senescence for all selected genes *in vitro*, but for *ADAMTS1* we did, which supports our *in vivo* observations. Of course, *in vitro* experiments do not completely reflect the *in vivo* situation. Furthermore, it is known that

different senescence inducers can lead to different senescent phenotypes, which may differ in disease context as well. We used our Paraquat model as this induces senescence by an increase in oxidative stress, likely the main contributor to senescence in COPD (as described above). In addition, the observed correlations *in vivo* may also reflect an indirect effect of accumulating senescent cells and their effect on the surrounding lung tissue, and thus may be different from senescence induction in a single cell type, like our *in vitro* model. Another possibility is that some of the observed correlations are driven by senescence in other cell types. Finally, in our *in vitro* model we only assessed the effect of senescence on ECM-related changes, but it should be kept in mind that altered ECM composition, structure and organization may also influence cellular senescence, as reviewed by Blokland et al [40].

In conclusion, after demonstrating a link between senescence and ECM dysregulation in COPD-derived lung fibroblasts previously, we have now demonstrated that many (SEO-)COPD-associated ECM-related genes and proteins are correlated with markers of senescence *in vivo* in lung tissue. As these ECM-related proteins include mainly regulators of ECM organization, proteases, and elastogenesis genes, our findings suggest that senescence can contribute to ECM dysregulation in COPD by affecting the protease-antiprotease imbalance and the disturbance of elastic fibres. This effect may partly be caused by senescent stromal cells, including fibroblasts, as some of the correlations were validated at single cell level and by *in vitro* experiments. The exact mechanism of how senescent cells can affect the protease-antiprotease imbalance and elastic fibre regulation and contribute to tissue remodelling in COPD should be investigated in more detail. This will lead to a better understanding of the functional consequences of increased senescence on disease pathology in COPD patients and may open new opportunities to develop therapeutic strategies to restore ECM regulation in COPD by targeting senescence.

Acknowledgements

We thank Marjan Reinders-Luinge and Wierd Kooistra (Department of Pathology and Medical Biology, University of Groningen, University Medical Centre Groningen) for processing the lung tissue and for isolation of the primary parenchymal lung fibroblasts from lung tissue from patients and subjects. We thank Anja Bakker (Department of Pathology and Medical Biology, University of Groningen, University Medical Centre Groningen) for her contribution to the measurements of secreted proteins using ELISA.

Funding

Aspasia, NWO (number: 015.015.044)
PPP-allowance by Top Sector Life Sciences & Health (Health Holland) and the University Medical Centre Groningen.

Disclosures

Prof. Timens reports incidental consulting fees from Merck Sharp Dohme, Bristol-Myers-Squibb, and Altana, outside the submitted work. Prof. Timens reports to be board member of Dutch Society of Pathology and member of Council for Research and Innovation of the Federation of Medical Specialists, outside the submitted work. All other authors have nothing to disclose.

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FIGURE LEGENDS

Figure 1: (SEO-)COPD-associated ECM-related genes and proteins in lung tissue. *ECM-related gene expression and protein levels were compared between COPD and controls (A+B) and SEO-COPD and controls (C+D) using transcriptomic (A+C) and proteomic (B+D) analyses. Volcano plots depict the LogFC (X-axis) and FDR (P adjust, Y-axis) of all detected ECM-related genes and proteins. Significant (P adjust < 0.05) differentially expressed ECM-related genes and proteins are depicted in colour, with higher expression in COPD in red and lower expression in COPD in blue. Horizontal dotted lines represent significance cut-off (FDR = 0.05), and vertical dotted lines represent LogFC of 0.*

Figure 2: Correlations between senescence genes and (SEO-)COPD-associated ECM-related genes and proteins. *All (SEO-)COPD associated ECM-related genes (A) and proteins (B) were correlated with the 6 major senescence genes using Pearson's correlations. The heatmaps depict the significant correlations (FDR < 0.05) in colour and the non-significant correlations in grey. The colours represent the strength (r coefficient) of the correlations, with positive correlations in red and negative correlations in blue. The ECM-related genes and proteins higher expressed in COPD are depicted on top and the ones lower expressed in COPD are depicted on the bottom of the y-axis in alphabetic order, the black line indicates the separation. The 6 senescence genes are depicted on the X-axis, in alphabetic order, with LMNB1 at last, as it is lower expressed in senescent cells.*

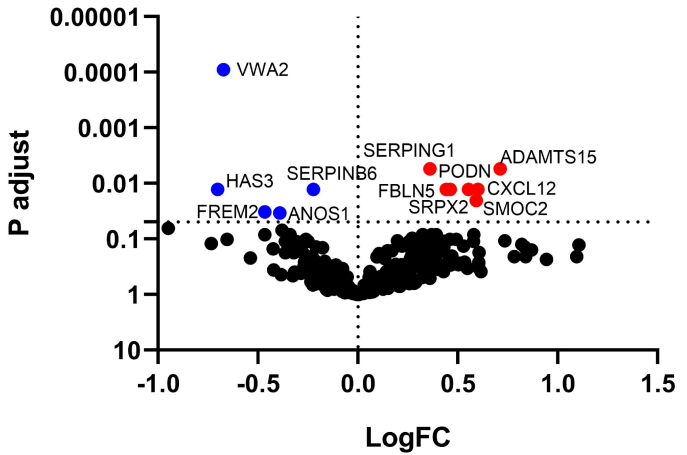
Figure 3: Strongest COPD-associated ECM and senescence correlations. *The strongest (SEO-)COPD-associated ECM and senescence correlations on transcript level, ADAMTS4, THBS1 and ADAMTS1, (A-C) and protein (FBLN5) level (D), in lung tissue are shown in scatter plots. Gene expression of p21 (CDKN1A) is depicted on x-axis and gene expression of ECM-related genes and protein is depicted on y-axis. The Pearson correlation coefficients (r) are depicted in the top left corner of the graphs.*

Figure 4: Correlations between p21 and ECM-related genes in stromal cells at single cell level. *The median ECM-related gene expression (log CPM) per population was depicted to visualize the population with the highest expression (A). The heatmap depicts the significant (P < 0.05) correlations between the COPD- and senescence-associated ECM with p21 in the stromal cell population in colour and the non-significant correlations in grey (B). The positive correlations are depicted in red, and the negative correlations are depicted in blue. The ECM-related genes and proteins higher expressed in COPD are depicted on top and the ones lower expressed in COPD are depicted on the bottom of the y-axis in alphabetic order, the black line indicates the separation. Examples of the 3 strongest significant COPD-associated ECM with p21 correlations on single cell level in stromal cells are depicted in scatter plots (C). The Spearman's correlation coefficients are depicted on the top left corner of the graphs.*

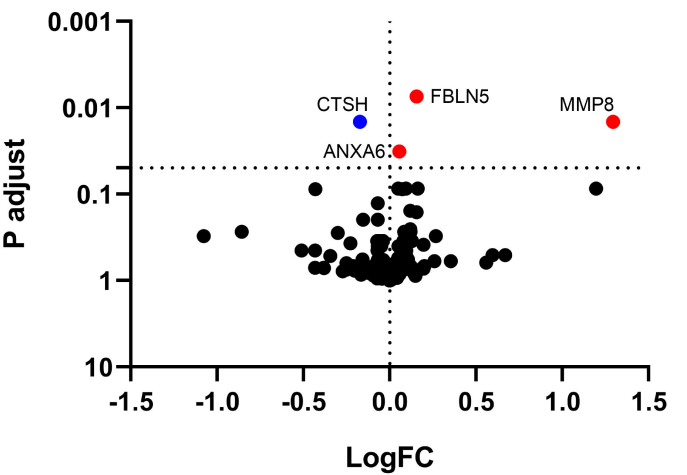
Figure 5: Functional validation of ECM-senescence correlations in senescence-induced lung fibroblasts. *Senescence was induced in primary lung fibroblasts (n=9) with 250uM PQ and after 5 days cells were reseeded to reach same confluency and gene expression and secreted protein levels were measured using RT-qPCR and ELISA, respectively, after 3 days of reseeding. Gene expression of ADAMTS4 (A), THBS1 (B), AND ADAMTS1 (C) of untreated (Basal, blue) and PQ-induced senescent (PQ, red) fibroblasts are shown in the dot plots. Relative gene expression was calculated using POLR2A as housekeeping gene and depicted. Secreted protein levels of THBS1 (D) and ADAMTS1 (E) are shown in the dot plots below. The lines represent matching donors. Statistical significance was tested using Wilcoxon signed-rank test, p-values are indicated and bold when < 0.05.*

Figure 6: FBLN5 protein level changes in senescence induced lung fibroblasts. *Senescence was induced in primary lung fibroblasts (n=9) with 250uM PQ and after 5 days cells were reseeded to reach same confluency. FBLN5 protein levels were measured using Western Blot after 3 days of reseeding. Examples of representative Western Blot membranes with visualization of full FBLN5, cleaved FBLN5 and Actin are shown in A. L = protein ladder, C = untreated fibroblasts & PQ = PQ-induced senescent fibroblasts. Quantification of total intensity of Full size FBLN5, Cleaved FBLN5 and the ratio of Cleaved FBLN5/Full size FBLN5 of untreated (Basal, blue) and PQ-induced senescent (PQ, red) fibroblasts are shown in the dot plots (B). The lines represent matching donors. Technical outliers were excluded. Statistical significance was tested using Wilcoxon signed-rank test, p-values are indicated and bold when < 0.05.*

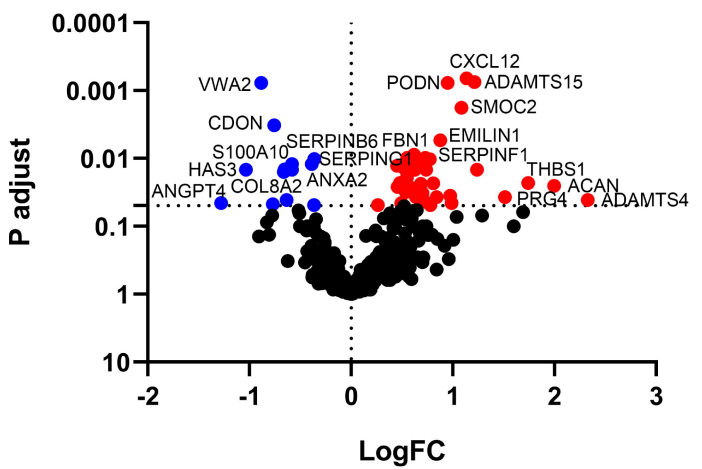
A

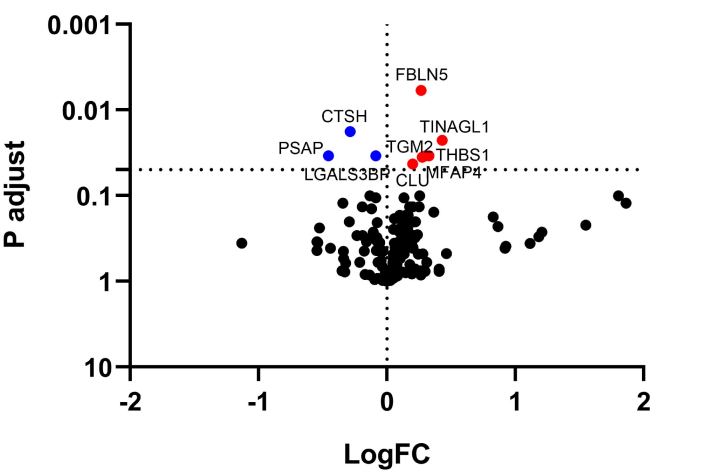

12 COPD-associated ECM genes
P adjust
LogFC
0.00001
0.0001
0.001
0.01
0.1
1
10
-1.0
-0.5
0.0
0.5
1.0
1.5
VWA2
HAS3
SERPINB6
SERPING1
ADAMTS15
PODN
FBLN5
CXCL12
SRPX2
SMOC2
FREM2
ANOS1

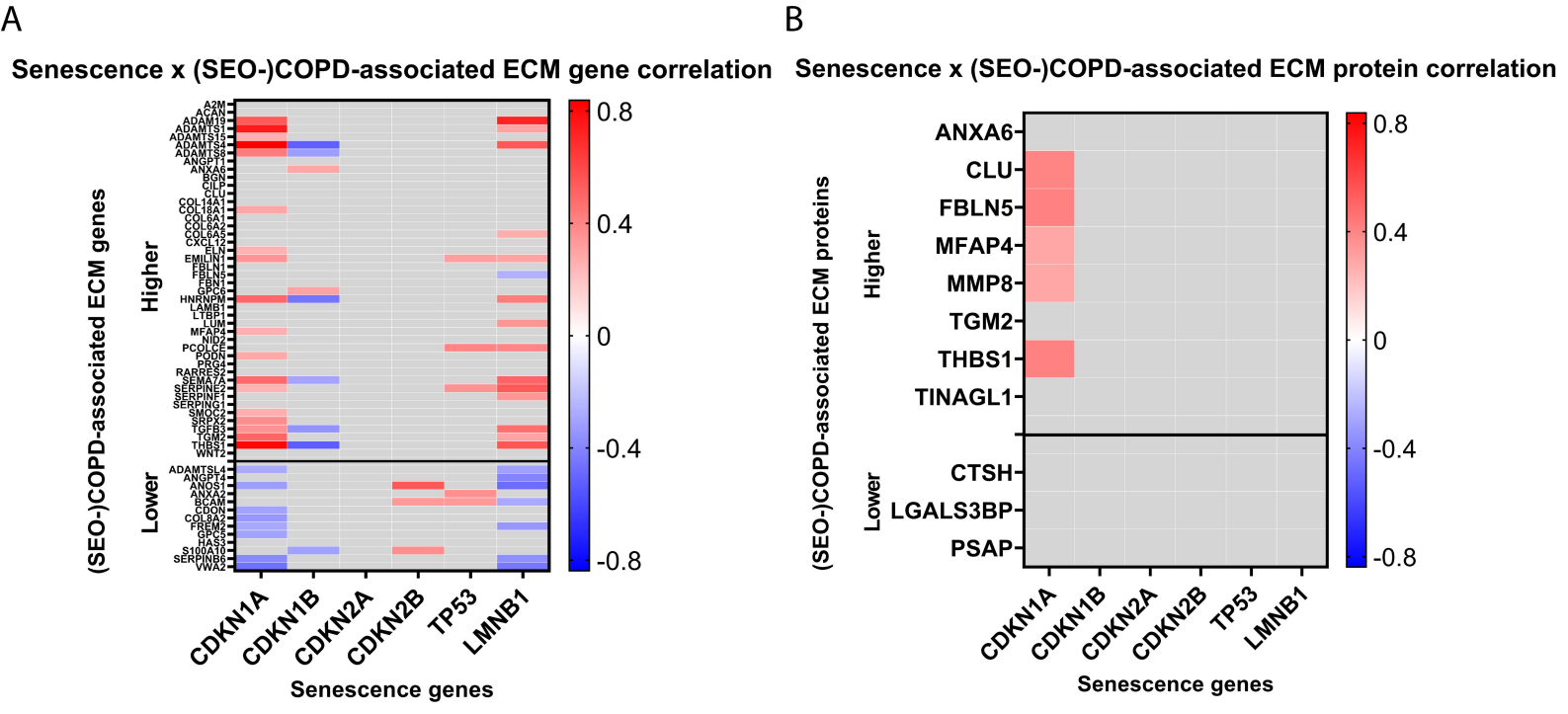
B

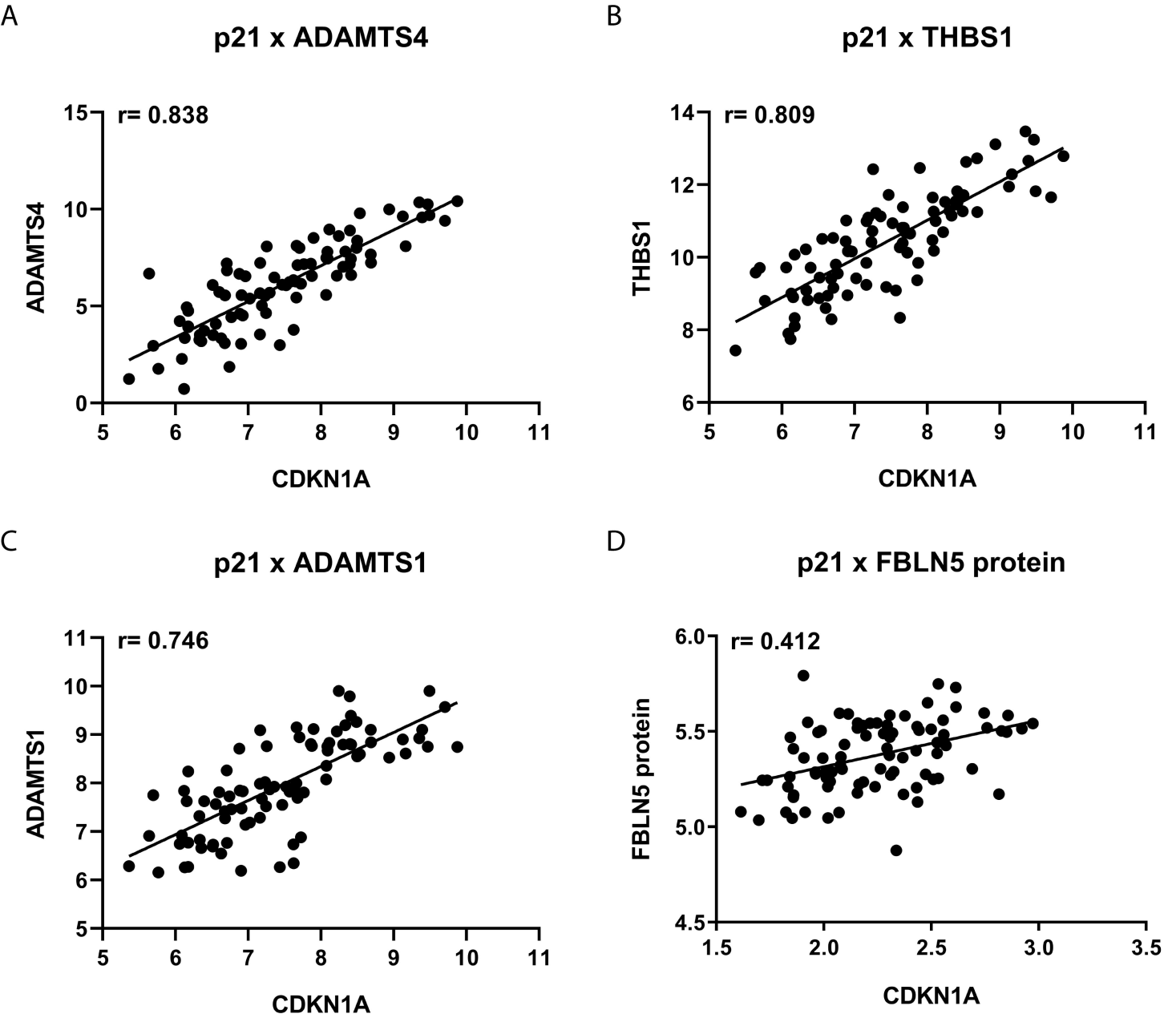

4 COPD-associated ECM proteins
P adjust
LogFC
0.001
0.01
0.1
1
10
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
CTSH
FBLN5
MMP8
ANXA6

C

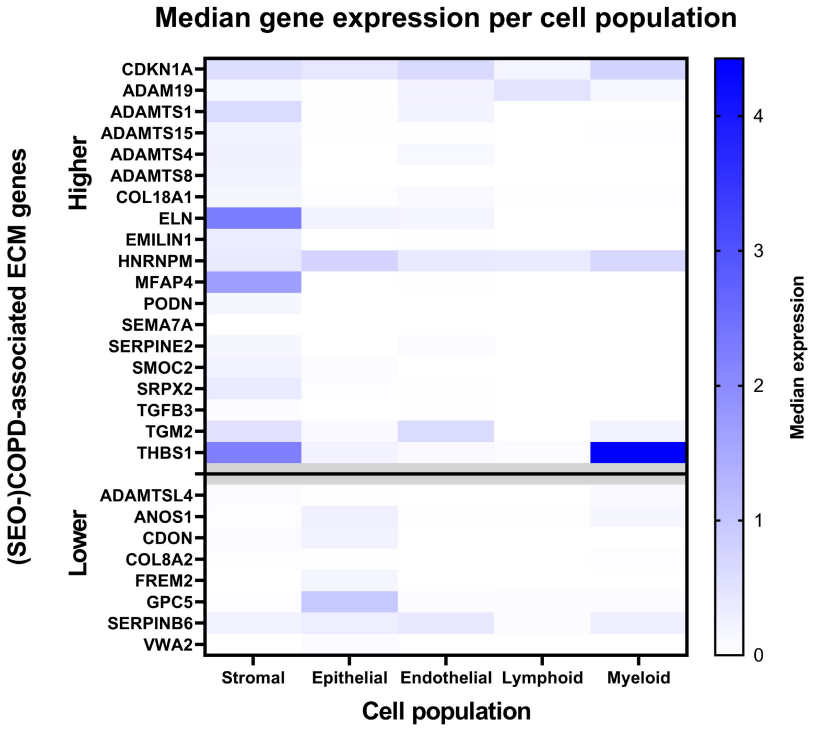

57 SEO-COPD-associated ECM genes
P adjust
LogFC
0.0001
0.001
0.01
0.1
1
10
-2
-1
0
1
2
3
VWA2
CXCL12
PODN
ADAMTS15
SMOC2
CDON
SERPINB6
FBN1
EMILIN1
S100A10
SERPING1
SERPINF1
HAS3
THBS1
ANXA2
ACAN
ANGPT4
COL8A2
PRG4
ADAMTS4


9 SEO-COPD-associated ECM proteins
P adjust
LogFC
0.001
0.01
0.1
1
10
-2
-1
0
1
2
FBLN5
CTSH
TINAGL1
PSAP
TGM2
THBS1
LGALS3BP
CLU
MFAP4

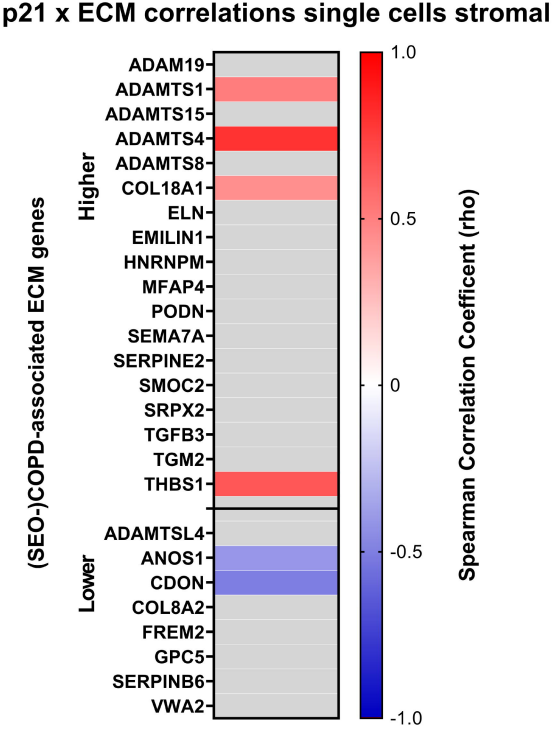

A
Senescence x (SEO-)COPD-associated ECM gene correlation
(SEO-)COPD-associated ECM genes
Higher
Lower
A2M
ACAN
ADAM17
ADAMTS1
ADAMTS16
ADAMTS4
ADAMTS9
ANGPT1
ANXA6
BGN
CILP
CLU
COL14A1
COL18A1
COL6A1
COL6A2
COL6A3
CXCL12
ELN
EMILIN1
FBLN1
FBLN5
FBN1
GPC4
HNRNPM
LAMB1
LTBP1
LUM
MFAP2
NID2
PCOLCE
PODN
PRG4
RARRES2
SEMA7A
SERPINE2
SERPINF1
SERPING1
SMOC2
SRPX2
TGFB3
TGM2
THBS1
WNT2
ADAMTSL4
ANGPT4
ANOS1
ANXA1
BCAM
CDON
COL8A2
FREM2
GPC3
HAS3
S100A16
SERPINB6
VWA2
0.8
0.4
0
-0.4
-0.8
CDKN1A
CDKN1B
CDKN2A
CDKN2B
TP53
LMNB1
Senescence genes
B
Senescence x (SEO-)COPD-associated ECM protein correlation
(SEO-)COPD-associated ECM proteins
Higher
Lower
ANXA6
CLU
FBLN5
MFAP4
MMP8
TGM2
THBS1
TINAGL1
CTSH
LGALS3BP
PSAP
0.8
0.4
0
-0.4
-0.8
CDKN1A
CDKN1B
CDKN2A
CDKN2B
TP53
LMNB1
Senescence genes


A
p21 x ADAMTS4
r= 0.838
ADAMTS4
CDKN1A
B
p21 x THBS1
r= 0.809
THBS1
CDKN1A
C
p21 x ADAMTS1
r= 0.746
ADAMTS1
CDKN1A
D
p21 x FBLN5 protein
r= 0.412
FBLN5 protein
CDKN1A

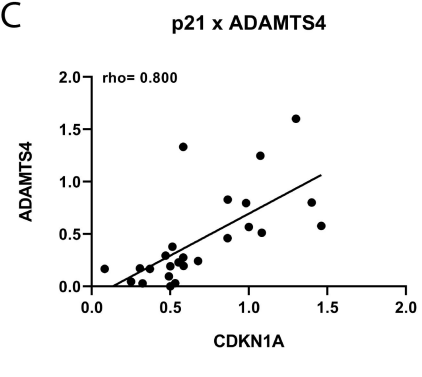
A

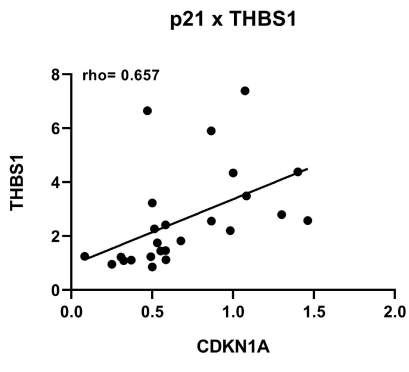

Median gene expression per cell population
(SEO-)COPD-associated ECM genes
Higher
Lower
CDKN1A
ADAM19
ADAMTS1
ADAMTS15
ADAMTS4
ADAMTS8
COL18A1
ELN
EMILIN1
HNRNPM
MFAP4
PODN
SEMA7A
SERPINE2
SMOC2
SRPX2
TGFB3
TGM2
THBS1
ADAMTSL4
ANOS1
CDON
COL8A2
FREM2
GPC5
SERPINB6
VWA2
Stromal
Epithelial
Endothelial
Lymphoid
Myeloid
Cell population
Median expression
0
1
2
3
4

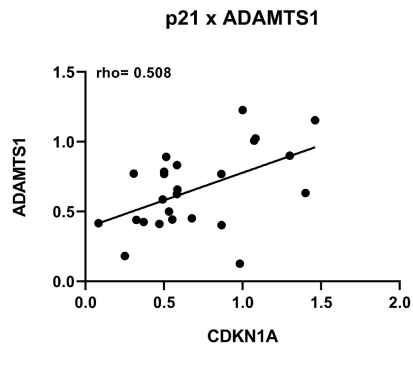
B


p21 x ECM correlations single cells stromal
(SEO-)COPD-associated ECM genes
Higher
Lower
ADAM19
ADAMTS1
ADAMTS15
ADAMTS4
ADAMTS8
COL18A1
ELN
EMILIN1
HNRNPM
MFAP4
PODN
SEMA7A
SERPINE2
SMOC2
SRPX2
TGFB3
TGM2
THBS1
ADAMTSL4
ANOS1
CDON
COL8A2
FREM2
GPC5
SERPINB6
VWA2
Spearman Correlation Coefficent (rho)
1.0
0.5
0
-0.5
-1.0

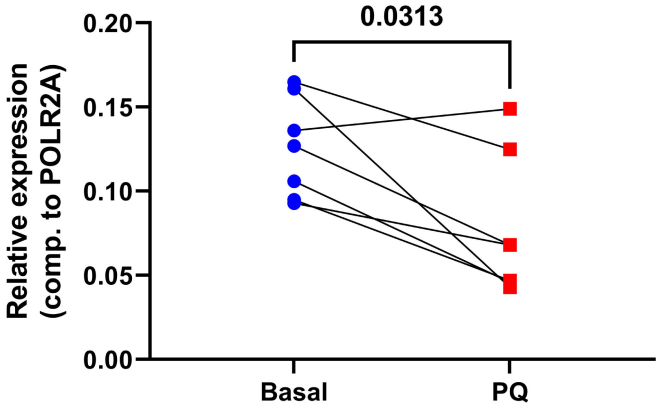
C


p21 x ADAMTS4
rho= 0.800
ADAMTS4
CDKN1A
0.0
0.5
1.0
1.5
2.0

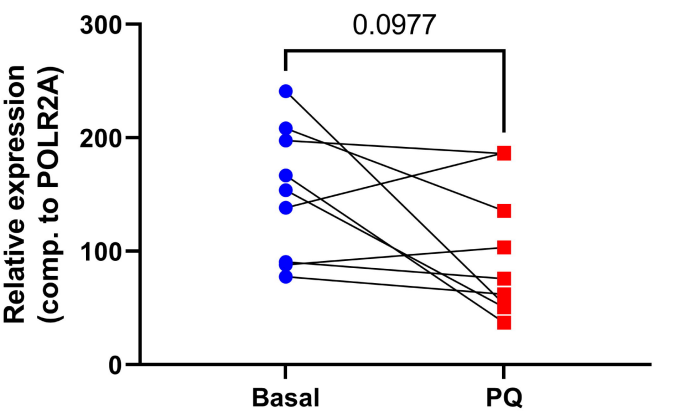

p21 x THBS1
rho= 0.657
THBS1
CDKN1A
0
2
4
6
8
0.0
0.5
1.0
1.5
2.0


p21 x ADAMTS1
rho= 0.508
ADAMTS1
CDKN1A
0.0
0.5
1.0
1.5
2.0

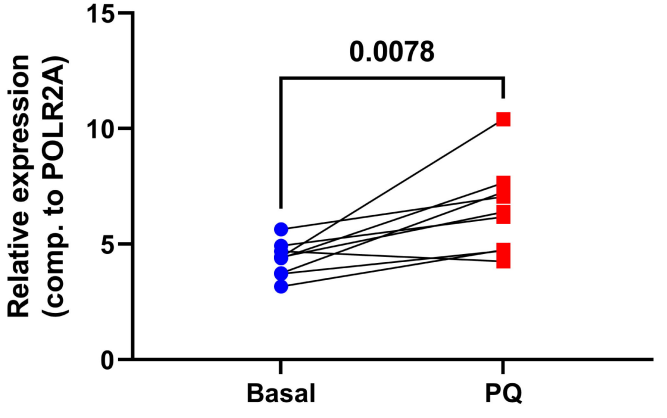
A


ADAMTS4 expression
0.0313
Relative expression (comp. to POLR2A)
0.20
0.15
0.10
0.05
0.00
Basal
PQ

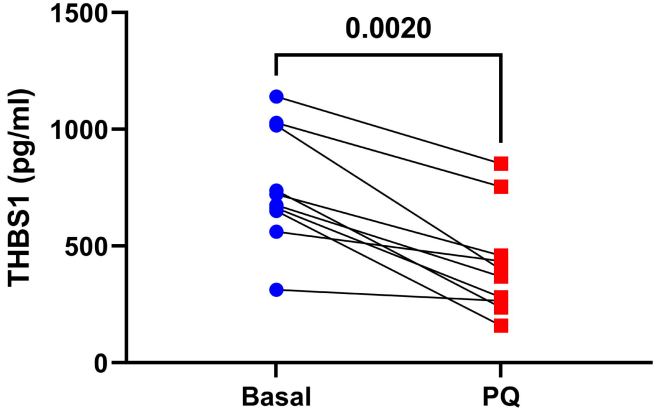
B


THBS1 expression
0.0977
Relative expression (comp. to POLR2A)
300
200
100
0
Basal
PQ

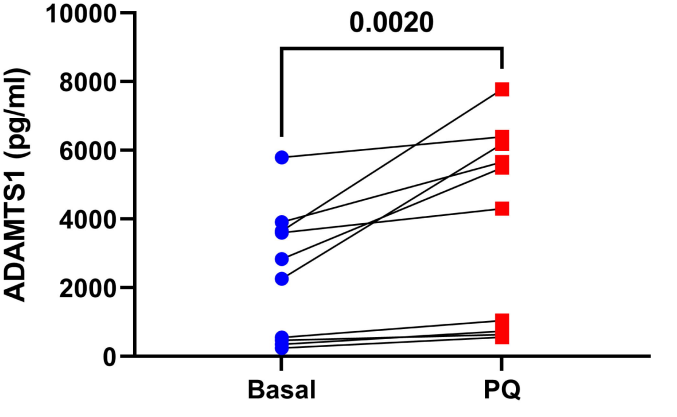
C

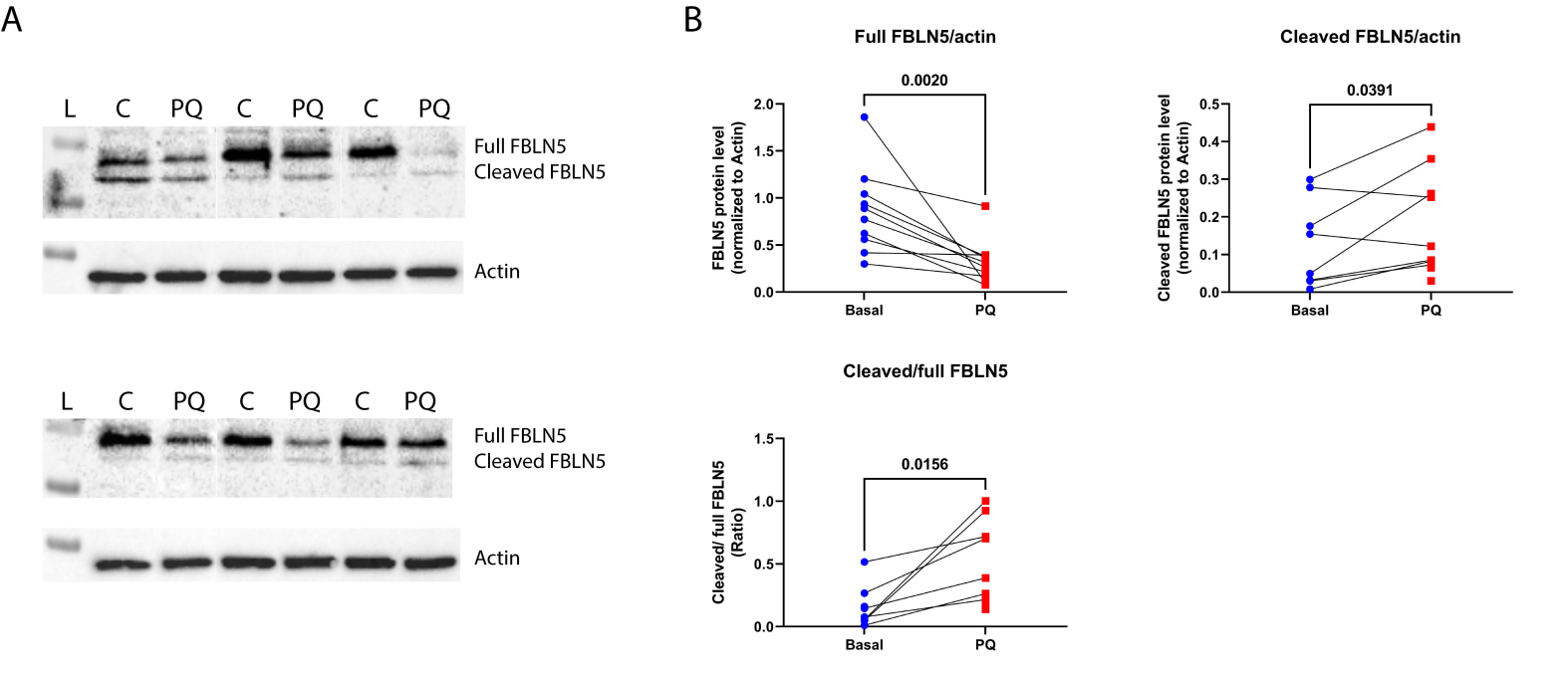

ADAMTS1 expression
0.0078
Relative expression (comp. to POLR2A)
15
10
5
0
Basal
PQ

D


THBS1 protein secretion
0.0020
THBS1 (pg/ml)
1500
1000
500
0
Basal
PQ

E


ADAMTS1 protein secretion
0.0020
ADAMTS1 (pg/ml)
10000
8000
6000
4000
2000
0
Basal
PQ


A
L C PQ C PQ C PQ
Full FBLN5
Cleaved FBLN5
Actin
L C PQ C PQ C PQ
Full FBLN5
Cleaved FBLN5
Actin
B
Full FBLN5/actin
0.0020
FBLN5 protein level (normalized to Actin)
2.0
1.5
1.0
0.5
0.0
Basal
PQ
Cleaved FBLN5/actin
0.0391
Cleaved FBLN5 protein level (normalized to Actin)
0.5
0.4
0.3
0.2
0.1
0.0
Basal
PQ
Cleaved/full FBLN5
0.0156
Cleaved/ full FBLN5 (Ratio)
1.5
1.0
0.5
0.0
Basal
PQ