Early View

Original article

Increased myofibroblasts in the small airways and relation to remodelling and functional changes in smokers and COPD patients: potential role of epithelial-mesenchymal transition (EMT)

Mathew Suji Eapen, Wenying Lu, Tillie L. Hackett, Gurpreet Kaur Singhera, Malik Q. Mahmood, Ashutosh Hardikar, Chris Ward, Eugene Haydn Walters, Sukhwinder Singh Sohal

Increased myofibroblasts in the small airways and relation to remodelling and functional changes in smokers and COPD patients: potential role of epithelial-mesenchymal transition (EMT)

Mathew Suji Eapen[1], Wenying Lu[1], Tillie L. Hackett[2a], Gurpreet Kaur Singhera[2b], Malik Q Mahmood[3], Ashutosh Hardikar[4,1], Chris Ward[5], Eugene Haydn Walters[6*], Sukhwinder Singh Sohal[1*#]

*Equal contributors

[1]Respiratory Translational Research Group, Department of Laboratory Medicine, School of Health Sciences, College of Health and Medicine, University of Tasmania, Launceston, Tasmania 7248, Australia.

[2a]Department of Anesthesiology, Pharmacology & Therapeutics, [2b]Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada, and UBC Centre for Heart Lung Innovation, St. Paul's Hospital, Vancouver, British Columbia BC V6Z 1Y6, Canada.

[3]School of Medicine, Deakin University, Waurn Ponds, Australia.

[4]Department of Cardiothoracic Surgery, Royal Hobart Hospital, Hobart, Tasmania 7000, Australia.

[5]Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, Tyne and Wear, UK.

[6]School of Medicine, and Menzies Institute of Medical Research, College of Health and Medicine, University of Tasmania, Hobart, Tasmania, 7000, Australia

[#]Corresponding Author

Dr Sukhwinder Singh Sohal

Respiratory Translational Research Group

Department of Laboratory Medicine, School of Health Sciences,

College of Health and Medicine, University of Tasmania

Locked Bag – 1322, Newnham Drive

Launceston, Tasmania 7248, Australia

Telephone number: +61 3 6324 5434

Email: sssohal@utas.edu.au

Abstract

Introduction: Previous reports showed epithelial mesenchymal transition (EMT) as an active process that contributes to small airway (SA) fibrotic pathology. Myofibroblasts are highly active pro-fibrotic cells that secrete excessive and altered extracellular matrix (ECM). Here we relate SA myofibroblast presence with airway remodelling, physiology and EMT activity in smokers and COPD patients.

Methods: Lung resections from non-smoker controls (NC), normal lung function smokers (NLFS), COPD current (CS) and ex-smokers (ES) were stained with anti-human αSMA, collagen 1, and fibronectin. αSMA+ive cells were computed in reticular basement membrane (Rbm), lamina propria (LP), and adventitia and presented per mm of Rbm and mm^2 of LP. Collagen-1 and fibronectin are presented as a percentage change from normal. All analysis including airway thickness were measured using Image-pro-plus 7.0.

Results: We found an increase in sub-epithelial LP (especially) and adventitia thickness in all pathological groups compared to NC. Increases in αSMA+ive myofibroblasts were observed in sub-epithelial Rbm, LP, and adventitia in both the smoker and COPD groups compared to NCs. Further, the increase in the myofibroblast population in the LP was strongly associated with decrease in lung function, LP thickening, increase in ECM protein deposition, and finally EMT activity in epithelial cells.

Conclusions: This is the first systematic characterization of small airway myofibroblasts in COPD based on their localization, with statistically significant correlations between them and other pan-airway structural, lung function, and ECM protein changes. Finally, we suggest that EMT may be involved in such changes.

Introduction

The GOLD has defined COPD as a disease state characterized by airflow limitation that is not entirely reversible. The airflow limitation is usually both progressive and associated with an abnormal (inflammatory) response of the `lungs` to noxious particles or gases" (1), by far the most common of these in Western countries being cigarette smoke. The major pathologic changes of COPD are observed in the airways, and functionally are in fact mainly fibrosis and destruction in the small airways (SA) wall (2). Emphysema of the peri-bronchial lung parenchyma occurs about 10 years after SA small airway obstruction can be detected (3, 4), and predominantly in these areas initially affected by air trapping (5).

In spite of an existing dogma that the airway wall is "inflamed" in COPD, the most comprehensive study on this, from our lab, demonstrated relative hypocellularity in the airway walls of both large and small airways, but in both areas the 'stromal' (mesenchymal) cell "fibroblast"-like population was the largest cell component (6). In the current study, we wished to further relate changes in a sub-population of these stromal cells, namely myofibroblasts, to airway wall thickening and deposition of strategic representative ECM proteins. Our focus was on SA tissue for these analyses as this is the predominant site of functional airflow changes in COPD. Because of this we have strong emphasised the relationship of these pathological small airway changes to appropriate measures of airflow obstruction (7).

Myofibroblasts are motile and contractile cells, with a high expression of alpha-smooth muscle actin (αSMA) protein. Previous studies in COPD evaluating expression of this protein marker for myofibroblasts in resected human airway tissue have been variable (8) in large and SA tissue. In contrast, findings from invitro studies with fibroblasts isolated from the distal end of the airways from COPD patients did show increased contractile properties associated with increased myofibroblast numbers (9). These findings suggest that myofibroblasts may be important in both large and small airways.

In the current study, we have used anti-αSMA antibody immunochemistry to identify and quantify the SA wall myofibroblast population, taking care to dissociate them from smooth muscle cell bundles. We have also descriptively analyzed

the localization of these cells in the SA wall tissue sub-layers. As mentioned, we have also analyzed whether the changes in these cell types have likely direct implications for airflow limitation in COPD through airway wall tissue remodelling, thickening and "scarring", i.e. re-organization of the ECM. Further, we also wished to evaluate further here whether these remodelling changes in the SA of smokers and COPD patients are likely to be driven by the presence of SA EMT activity that we have previously published as being related to airflow obstruction in COPD (10-13).

Materials and methods

Ethics Approvals

The Tasmanian Health & Medical Human Research Ethics Committee approved the study (H0012374). Tissues from normal non-smoker controls were obtained from the James Hogg Lung Registry, the University of British Columbia with approval from the Providence Health Care Research Ethics Board (H00–50110).

Subject Demographics

Resected tissues containing multiple SA suitable for analysis (<2mm internal diameter) from forty patients were included (Table 1). These non-normal subjects all had primary non-small cell lung cancer, with an approximately equal distribution of squamous- and adenocarcinoma. Twenty patient demonstrated mild-moderate GOLD stage I or II, of whom nine were COPD-CS and eleven COPD-ES (>1year smoking cessation). Eleven individuals were NLFS. Tissue from ten NC obtained from the tissue bank at the University of British Columbia. Subjects with other respiratory diseases, a history of a recent acute exacerbation of COPD and those on systemic or inhaled corticosteroids were excluded from the study. The surgically resected material was taken well away from the primary tumour and contained no cancer-involved SA or related pneumonitis.

Immunostaining

Sections were cut at 3-microns from individual paraffin-embedded blocks. Immunostaining for mouse monoclonal anti-αSMA (Dako, catalog # M0851, 1:400 dilution), mouse polyclonal anti-collagen-1 (Abcam catalog # AB34710, 1:250 dilution) polyclonal rabbit fibronectin (Dako, A0245, 1:1000 dilution) respectively, for

90 min at room temperature. Species-appropriate isotype-matched immunoglobulin G (X0931 clone DAK-GO1; Dako) was incorporated. Bound antibodies were elaborated using peroxidase-labeled Envision (catalogue number K4001; Dako) and DAB (catalogue number K3468; Dako). We also incorporated an overlapping group of smoker and COPD tissues from a previous study (14) in which SAs had been stained in the same way as above for S100A4 and vimentin,both EMT activity markers in epithelium, where they are co-expressed with epithelial proteins.

Quantification of SA tissue staining

Image analysis was performed with a Leica DM 2500 microscope (Leica Microsystems, Germany), Spot Insight-12 (Spot Imaging Solutions, USA) digital camera and Image Pro Plus 7.0 (Media Cybernetics, USA) software. Firstly, as many images as possible were taken of the airway wall from multiple areas. Study included epithelium plus sub-mucosal layers down to the alveolar interface, and strictly avoided overlapping of tissue. For each of the measurements, eight randomly selected images including the full airway thickness from at least eight good pictures.

Assessing SA wall thickness

For study of SA thickness approximately three to four small airways per patient were analyzed, and of the total images, eight were selected using an online random number generator programme (www.stattrek.com). These tissue pictures were each divided into three sub-epithelial regions: the lamina propria (the area between the lower limit of the Rbm and the upper margin of the muscle layer); the smooth muscle layer; and the adventitia (the area between lower margin of the muscle to the margin of the alveolar tissue interface) (figure 1 a and b). Using Image ProPlus version 7.0 software tools, for each layer thickness a line was drawn at each extreme layer margins, i.e. at the interface with the one above and below. Based on tissue orientiation, either the horizontal, vertical or curved tool was selected, and the average distance between the margins was calculated using an automated distance calculator programme within the Image ProPlus 7.0 software.The analysis was conducted by an observer (MSE) who was blinded to subject and clinical group.

Quantification of myofibroblasts and ECM proteins

αSMA-positive cells with a fibroblast-like morphology were classified as myofibroblasts and enumerated in the SA wall Rbm, lamina propria, and adventitia regions. Such αSMA-positive cells in the Rbm were enumerated as cells per mm length of the Rbm, while for the lamina propria and adventitia the cells were enumerated as per mm^2 of the respective area. For the expression of ECM "scar" proteins collagen-1 and fibronectin, diffused brown staining was selected in the area of interest (AOI) drawn manually for both LP (the area between the lower limit of the Rbm and the upper margin of the muscle layer) and adventitia (the area between lower margin of the muscle to the margin of the alveolar tissue interface) regions using tissue analysis software Image ProPlus 7.0 Further, the software was used to generate a ratio of the collagen-1 and fibronectin staining in each selected AOI, which is presented here as "percent staining". All analyses were conducted by an observer (MSE) who was blinded to clinical group and other tissue pathology measures.

Statistical analysis

For all cross-sectional data, we tested their normal distributions using the D'Agostino-Pearson omnibus normality test. Non-parametric analyses of variance were performed using the Kruskal-Wallis Test, which compared medians/ranges across all the groups of interest; specific group differences with correction for multiple comparisons were assessed using Dunn's test. For correlations, we performed regression analyses using Spearman's rank test. These statistical analyses were done using GraphPad Prism V8.0, with a p-value ≤0.05 being considered significant.

Results

Increased SA wall component thickness in smokers and COPD

Both the LP and adventitia were significantly thicker in COPD subjects, with the LP showing at least a 10-fold change overall compared to normal controls whereas in the adventitial areas there was a 2 to 3-fold thickening (figure 1). The muscle layer too was thickened, but only by 50% (figure 1e). All these changes together were

reflected in a substantial increase in total SA thickness in COPD, but also in smokers almost equally except in the LP where there appeared to be some interaction between active smoking and COPD.

Airway thickening versus airflow measures

There was a significant negative correlation between increased SA wall thickening and decrease in airflow-related lung function in the COPD groups combined, but only with LP sub-epithelial layer (Figure 2 a,b).

αSMA-positive myofibroblast in SA.

Significant increases in SA αSMA+ myofibroblasts were observed throughout the airway wall in the smokers and COPD groups, but most consistently in the latter. The density and increase in myofibroblasts were especially more striking in the LP and in actively smoking COPD. The increases in myofibroblast numbers in the SA LP of smokers/COPD tissues were not uniform, but the cells were most concentrated in a shallow band just deep to the Rbm (Figure 3).

ECM deposition in SA wall

There was an overall increase in the key ECM proteins, collagen-1 and fibronectin in the airway wall in all three smoker and COPD groups, with fibronectin changes being the most pronounced (Figure 4). Empirically there were both smoking and COPD effects, with greatest effects seen in the COPD-CS group. In comparison to percent collagen-1 expression in the LP (1.5-fold increase), the increase in the adventitia was much greater (5-6 fold) in smokers and COPD patients compared to NC (Figure 4 a, c). This distribution difference was not seen for fibronectin where the fold increase was more uniform (Figure 4 b, d).

Correlation of SA myofibroblasts with airflow physiologic measures, and with SA wall LP thickening

We present a complex set of correlations to show together the relationships between myofibroblast numbers in the Rbm and the LP against airflow in the three clinical groups. Although group numbers are rather small, there was still a significant or near significant correlation between myofibroblast numbers versus decreases in airflow (obstruction), both as FEV1/FVC% and FEF25-75%, in both COPD groups but not in

the NLFS (Figure 5 a ,b, c and d). Again, relationships were most consistently seen between airflow obstruction and tissue myofibroblasts in the currently smoking COPD group. Regression analysis between myofibroblast density in the SA LP region and LP thickness in all pathological groups (including current and ex-smokers) (Figure 5e) showed a very similar picture to the above but suggesting both a smoking and COPD effect.

Correlation of percent Collagen-1 and Fibronectin with lung function and SA wall LP thickness in smoker/COPD groups.

A significant correlation was seen between collagen-1 deposition in the SA LP and lung function in the COPD-CS, but this was absent in the ex-smoker group. However, no significant correlation was found between fibronectin deposition in SA LP or lung function (Table 2). The percent by area tissue for collagen-1 expression in the LP was significant and positively correlated with LP thickness in COPD (both CS and ES) patients, but in contrast this was not the case in the normal lung function smokers although their LP thickness was increased as already shown (Table 2). Notably, and contrary to collagen-1 expression, fibronectin percentage area positivity correlated to increasing normal smoker LP thickness only and not in the COPD groups (Table 2).

Correlation of EMT markers in SA wall cells versus number of αSMA+ myofibroblasts and airway thickening.

EMT-marker expression in basal epithelial cells showed a postive association with αSMA positive myofibroblasts in Rbm (Figure 6a and b) of the SA wall in combined COPD, and NLFS groups with a ratio of approx 4 to 1. Within the Rbm there was also a relationship between S100A4 and αSMA+ive cells with a ratio of approx 1.5 to 1, i.e. suggesting a transition of mesenchymal-marker positive cells towards myofibroblasts, with αSMA cell expression thought to represent a late manifestation of the EMT process. Equally notable, there was also a positive relationship between basal epithelial cell EMT activity as indicted by both S100A4 and vimentin expression and the (enhanced) thickness of the LP.

Discussion

A core finding in this study was the increase in αSMA-positive myofibroblasts in the SA wall of COPD patients and its association with a major increase in the LP sub-layer thickness. There were also increases in thickness of the Rbm, muscle layer and adventitia. These changes in the myofibroblast population were also directly related to significant pathological changes in the ECM "scar" proteins, collagen-1 and fibronectin. In context to our earlier findings of EMT activity in SA epithelium in smokers and COPD, we believe that such transformation could lead to an increase in fibroblast or myofibroblast population. Both EMT activity and what we suggest as secondary changes in the myofibroblast population and consequential LP thickening were also related to obstructive airflow limitation. Thus, from our current and previous observations, we suggest that EMT may play a crucial role in the SA wall remodelling which leads to SA narrowing and ultimately obliteration, as we and others too have previously suggested (11, 13, 15, 16).

It has been accepted that one of the principal causes for airflow limitation in COPD is the airway wall tissue remodeling through re-organization of the ECM (17). In the current study, we analyzed two important markers of ECM pathology, collagen-1, and fibronectin, both of which have been described as co-localized to areas with increased proliferation of myofibroblasts in COPD (18, 19). Although we found significant increases in percent expression of both these ECMs, there were also marked differences between fibronectin and collagen-1 expression in various SA sub-layers. Further, both ECM protein expressions were increased in smokers but more so in COPD-CS, i.e. both factors seemed to have influence in disease progression. Understanding potential consequence of these anatomic variations will need further effort, but we found collagen1 but not fibronectin in LP was associated with airflow obstruction in COPD. For fibronectin, its cellular immune-modulatory roles may be more important than structural ones (20).

Our current observation of an increase in collagen-1 is in agreement with the earlier studies done by Harju et al (19) in SA tissues where an overall increase in both collagen I and III subtypes in GOLD stages I and II COPD were observed in the SA LP. At the same time, both these studies contrast to observations by Annoni et al (21) who suggested a decrease in collagen-1 deposition in SA in mild-moderate

COPD patients, although they too found an increase in fibronectin in both smokers and COPD. Furthermore, ECM changes seemed to regress in COPD-ES, and indeed relationships to lung function were significant in current-smoking COPD but not ex-smokers. The cause of such regression since quitting would seem to be an important research question and needs wider attention in the research community.

ECM-producing myofibroblasts have a spindle-shaped morphology and are highly contractile due the presence of αSMA microfilaments (22, 23). Surprisingly, few reports are published so far that our current data could be compared to. Our finding of an increase in αSMA-positive myofibroblasts (on morphology criteria) is in contrast to Karvonen et al (24) who showed a decrease in expression αSMA-positive cells in the bronchioles of COPD patients compared to non-smoker controls. The differences in the findings are likely due to the counting strategy and area under consideration. Thus, while Karovnen et al (24) counted αSMA-positive cells in the whole of the sub-epithelial wall, in the current study we took each sub-layer in turn. Indeed, Karovnen et al considered the Rbm and the muscle layer as part of the LP for SA. Our findings, however, are similar to those of Harju et al (19) who provided a descriptive analysis of the SA tissue and evidence of co-localization of αSMA-positive cells with collagen subtypes as well as mesenchymal markers such as vimentin in the SA wall. They, like us, suggested that αSMA-positive myofibroblasts could be responsible for the increased accumulation of collagen-1 and fibronectin in the SA LP of COPD patients, and like us others have suggested myofibroblasts as being responsible for airway wall thickening secondary to ECM accumulation (supplementary figure 1) (19).

We have taken these matters substantially further than previous reports, observing physiological measures of reduced airway caliber with increasing myofibroblast density accompanying changes in ECM, and suggesting possible involvement of this cell type in SA remodeling and narrowing and ultimately obliteration (2, 25). We should emphasise, therefore, that the airways that we are studying are the "survivor" airways from this obliterative SA process, and we are looking at glimpses of pathogenic processes going on for many years in any one individual.

As mentioned, our group's previous data (14) suggested that the underlying mechanism for airway wall remodeling is through the induction of EMT (10, 26), as

part of a broader epithelial-gene re-programming (27, 28). We now suggest recruitment of myofibroblasts to the underlying airway wall from the mesenchymally transitioned basal stem cells of the epithelium. We have previously demonstrated a strong relationship between markers of epithelial EMT activity, such as S100A4 and vimentin expression in these basal cells, with increasing airway obstruction (10, 14). TGFβ1 pathways are likely to contribute to driving EMT in COPD, via nuclear transcription factors such as pSMAD2/3 with reduction in the inhibitory SMAD7 (12, 29). Interestingly, TGFβ1 pathways are also suggested to play a crucial role in the development of myofibroblasts from tissue fibroblasts through activation of the SMAD pathway (25, 30), so although we believe EMT to be a key mechanism in COPD pathogenesis (31) it is unlikely to be the only growth-factor-driven mechanism operating throughout the whole thickness of the SA wall. In addition to the TGFβ1 pathway we have previously shown along with others that the transcription factor clusters of β-catenin/Snail1/Twist is upregulated and with nuclear translocation in smokers and COPD, and their expression is closely related to both EMT activity and lung function (27, 32, 33).

There are limitations to our study. The number of individual subjects contributing tissue samples per clinical group were relatively small due to study logistics, but even so many of the findings are nevertheless statistically robust for the most part and consistently so, emphasizing the strength if the signals obtained. This study also included a wider age range in the normal control subjects with median age significantly lower than the pathological subjects (Table-1). As the age of the control subjects is significantly lower than the age of patients with COPD, we cannot exclude that our observations partly result from possible contribution of age in addition to smoking and disease. We did find that smokers with normal lung function had thick airway wall, increased myofibroblast numbers and ECM changes significantly higher compared to normal subjects and closer to levels of patients with COPD (34). Different studies have reported morphological changes in the airways with normal ageing, which mainly includes progressive decrease in cartilage thickening and airway dilation, but interestingly in CT image analysis of the small airways, no linear progression in airway wall thickening and aging was observed in normal subjects especially between the fourth-sixth generation airways, which were indeed thicker in patients with COPD (35, 36, 37). Clinical features such as air trapping seems to be

common between aging lung and COPD. Another generic problem in this method of tissue research, the current smoker/COPD SA were obtained from cancer patients and thus some confounding by this disease pathology and secondary pneumonitis could conceivably be present. However, all the tissue used was carefully taken under microscopy by an experienced pathologist well away from cancer-involved areas.

Conclusion

We know from the work of Hogg et al (7) and indeed earlier studies, that a large number of SA will have been obliterated, leaving just a scar, by the time a smoker has reached the degree of airflow obstruction that can be classified as COPD. Our regression analyses showed that despite the cumulative damage that has already occurred the activity of current processes still are likely to reflect the totality of this pathophysiology. Thus, our conclusions reflect not only acute pathology relevant to an arbitrary point in time for each individual when samples were obtained, but the totality of the data allows a quite profound representation of the whole history of the core pathological process in smoking-related COPD going back over many years. Our data would support the logic and need for treatment as early as possible and identifies possible new pathophysiological targets for therapy.

Conflict of interest: S. S. Sohal reports personal fees for lectures from Chiesi, outside the submitted work. All the other authors do not have any conflict of interest to declare.

References

1. Vestbo J, Hurd SS, Agustí AG, Jones PW, Vogelmeier C, Anzueto A, Barnes PJ, Fabbri LM, Martinez FJ, Nishimura M, Stockley RA, Sin DD, Rodriguez-Roisin R. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. *American journal of respiratory and critical care medicine* 2013; 187: 347-365.
2. Koo HK, Vasilescu DM, Booth S, Hsieh A, Katsamenis OL, Fishbane N, Elliott WM, Kirby M, Lackie P, Sinclair I, Warner JA, Cooper JD, Coxson HO, Paré PD, Hogg JC, Hackett TL. Small airways disease in mild and moderate chronic obstructive pulmonary disease: a cross-sectional study. *The Lancet Respiratory medicine* 2018; 6: 591-602.
3. Thurlbeck WM, Dunnill MS, Hartung W, Heard BE, Heppleston AG, Ryder RC. A comparison of three methods of measuring emphysema. *Human Pathology* 1970; 1: 215-226.
4. Eapen MS, Myers S, Walters EH, Sohal SS. Airway inflammation in chronic obstructive pulmonary disease (COPD): a true paradox. *Expert Review of Respiratory Medicine* 2017; 11: 827-839.
5. O'Donnell DE, Laveneziana P. Physiology and consequences of lung hyperinflation in COPD. *European Respiratory Review* 2006; 15: 61-67.
6. Eapen MS, McAlinden K, Tan D, Weston S, Ward C, Muller HK, Walters EH, Sohal SS. Profiling cellular and inflammatory changes in the airway wall of mild to moderate COPD. *Respirology* 2017; 22: 1125-1132.
7. Hogg JC, Paré PD, Hackett T-L. The Contribution of Small Airway Obstruction to the Pathogenesis of Chronic Obstructive Pulmonary Disease. *Physiol Rev* 2017; 97: 529-552.
8. Löfdahl M, Kaarteenaho R, Lappi-Blanco E, Tornling G, Sköld MC. Tenascin-C and alpha-smooth muscle actin positive cells are increased in the large airways in patients with COPD. *Respiratory Research* 2011; 12: 48-48.
9. Hallgren O, Rolandsson S, Andersson-Sjöland A, Nihlberg K, Wieslander E, Kvist-Reimer M, Dahlbäck M, Eriksson L, Bjermer L, Erjefält JS, Löfdahl C-G, Westergren-Thorsson G. Enhanced ROCK1 dependent contractility in fibroblast from chronic obstructive pulmonary disease patients. *J Transl Med* 2012; 10: 171-171.
10. Sohal SS, Reid D, Soltani A, Ward C, Weston S, Muller HK, Wood-Baker R, Walters EH. Reticular basement membrane fragmentation and potential epithelial mesenchymal transition is exaggerated in the airways of smokers with chronic obstructive pulmonary disease. *Respirology* 2010; 15: 930-938.

11. Sohal SS, Reid D, Soltani A, Ward C, Weston S, Muller HK, Wood-Baker R, Walters EH. Evaluation of epithelial mesenchymal transition in patients with chronic obstructive pulmonary disease. *Respiratory research* 2011; 12: 130.

12. Sohal SS, Soltani A, Reid D, Ward C, Wills KE, Muller HK, Walters EH. A randomized controlled trial of inhaled corticosteroids (ICS) on markers of epithelial-mesenchymal transition (EMT) in large airway samples in COPD: an exploratory proof of concept study. *International journal of chronic obstructive pulmonary disease* 2014; 9: 533-542.

13. Sohal SS, Walters EH. Role of epithelial mesenchymal transition (EMT) in chronic obstructive pulmonary disease (COPD). *Respir Res* 2013; 14: 120.

14. Mahmood MQ, Sohal SS, Shukla SD, Ward C, Hardikar A, Noor WD, Muller HK, Knight DA, Walters EH. Epithelial mesenchymal transition in smokers: large versus small airways and relation to airflow obstruction. *International journal of chronic obstructive pulmonary disease* 2015; 10: 1515-1524.

15. Sohal SS, Eapen MS, Ward C, Walters EH. Epithelial-Mesenchymal Transition: A Necessary New Therapeutic Target in Chronic Obstructive Pulmonary Disease? *American journal of respiratory and critical care medicine* 2017; 196: 393-394.

16. Sohal SS, Eapen MS, Ward C, Walters EH. Airway inflammation and inhaled corticosteroids in COPD. *European Respiratory Journal* 2017; 49: 1700289.

17. Ito JT, Lourenço JD, Righetti RF, Tibério IFLC, Prado CM, Lopes FDTQS. Extracellular Matrix Component Remodeling in Respiratory Diseases: What Has Been Found in Clinical and Experimental Studies? *Cells* 2019; 8: 342.

18. Stylianou P, Clark K, Gooptu B, Smallwood D, Brightling CE, Amrani Y, Roach KM, Bradding P. Tensin1 expression and function in chronic obstructive pulmonary disease. *Scientific Reports* 2019; 9: 18942.

19. Harju T, Kinnula VL, Pääkkö P, Salmenkivi K, Risteli J, Kaarteenaho R. Variability in the precursor proteins of collagen I and III in different stages of COPD. *Respiratory Research* 2010; 11: 165-165.

20. Thomas AH, Edelman ER, Stultz CM. Collagen fragments modulate innate immunity. *Experimental biology and medicine (Maywood, NJ)* 2007; 232: 406-411.

21. Annoni R, Lanças T, Yukimatsu Tanigawa R, de Medeiros Matsushita M, de Morais Fernezlian S, Bruno A, Fernando Ferraz da Silva L, Roughley PJ, Battaglia S, Dolhnikoff M, Hiemstra PS, Sterk PJ, Rabe KF, Mauad T. Extracellular matrix composition in COPD. *European Respiratory Journal* 2012; 40: 1362-1373.

22. Murray LA, Knight DA, Laurent GJ. Chapter 15 - Fibroblasts. In: Barnes PJ, Drazen JM, Rennard SI, Thomson NC, editors. Asthma and COPD (Second Edition). Oxford: Academic Press; 2009. p. 193-200.

23. Pellegrin S, Mellor H. Actin stress fibres. *Journal of Cell Science* 2007; 120: 3491-3499.

24. Karvonen HM, Lehtonen ST, Harju T, Sormunen RT, Lappi-Blanco E, Mäkinen JM, Laitakari K, Johnson S, Kaarteenaho RL. Myofibroblast expression in airways and alveoli is affected by smoking and COPD. *Respiratory Research* 2013; 14: 84-84.

25. Gu L, Zhu YJ, Yang X, Guo ZJ, Xu WB, Tian XL. Effect of TGF-beta/Smad signaling pathway on lung myofibroblast differentiation. *Acta pharmacologica Sinica* 2007; 28: 382-391.

26. Crystal RG. Airway basal cells. The "smoking gun" of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* 2014; 190: 1355-1362.

27. Mahmood MQ, Walters EH, Shukla SD, Weston S, Muller HK, Ward C, Sohal SS. β-catenin, Twist and Snail: Transcriptional regulation of EMT in smokers and COPD, and relation to airflow obstruction. *Scientific Reports* 2017; 7: 10832.

28. Sohal SS. Airway Basal Cell Reprogramming and Epithelial-Mesenchymal Transition: A Potential Key to Understanding Early Chronic Obstructive Pulmonary Disease. *American journal of respiratory and critical care medicine* 2018; 197: 1644-1645.

29. Mahmood MQ, Reid D, Ward C, Muller HK, Knight DA, Sohal SS, Walters EH. Transforming growth factor (TGF) β_1 and Smad signalling pathways: A likely key to EMT-associated COPD pathogenesis. *Respirology* 2017; 22: 133-140.

30. Harris WT, Kelly DR, Zhou Y, Wang D, MacEwen M, Hagood JS, Clancy JP, Ambalavanan N, Sorscher EJ. Myofibroblast differentiation and enhanced TGF-B signaling in cystic fibrosis lung disease. *PLoS One* 2013; 8: e70196.

31. Eapen MS, Sohal SS. Update on the Pathogenesis of COPD. *The New England journal of medicine* 2019; 381: 2483-2484.

32. Eapen MS, Sohal SS. WNT/β-catenin pathway: A novel therapeutic target for attenuating airway remodelling and EMT in COPD. *EBioMedicine* 2020; 62: 103095.

33. Carlier FM, Dupasquier S, Ambroise J, Detry B, Lecocq M, Biétry-Claudet C, Boukala Y, Gala JL, Bouzin C, Verleden SE, Hoton D, Gohy S, Bearzatto B, Pilette C. Canonical WNT pathway is activated in the airway epithelium in chronic obstructive pulmonary disease. *EBioMedicine* 2020; 61: 103034.

34. Telenga ED, Oudkerk M, van Ooijen PM, Vliegenthart R, Ten Hacken NH, Postma DS, van den Berge M. Airway wall thickness on HRCT scans decreases with age and increases with smoking. BMC Pulm Med. 2017 Feb 1;17(1):27.

35. Su ZQ, Guan WJ, Li SY, Feng JX, Zhou ZQ, Chen Y, Zhong ML, Zhong NS. Evaluation of the Normal Airway Morphology Using Optical Coherence Tomography. Chest. 2019 Nov;156(5):915-925.

36. Bommart S, Marin G, Bourdin A, Revel MP, Klein F, Hayot M, Vachier I, Chanez P, Picot MC, Mercier J, Molinari N, Vernhet-Kovacsik H. Computed tomography quantification of airway remodelling in normal ageing subjects: a cross-sectional study. Eur Respir J. 2015 Apr;45(4):1167-70.

37. Occhipinti M, Larici AR, Bonomo L, Incalzi RA. Aging Airways: between Normal and Disease. A Multidimensional Diagnostic Approach by Combining Clinical, Functional, and Imaging Data. Aging Dis. 2017 Jul 21;8(4):471-485.

Table-1

Patient demographic details.

Study groups	NC	NLFS	COPD-CS	COPD-ES
n	10	11	9	10
Age(years)	50 (19-73)	69 (52-79) **	65 (59-78) **	68 (56-85) **
Smoking history (Pack-years)	NS	27 (0.3-40)	30 (2-48)	30 (2-58)
FEV_1/FVC (%)*	N/A	76 (70-90)	66 (59.9-70)	64 (55-69)
$FEF_{25-75\%}$ *	N/A	79 (47-116)	37 (28-47)	41 (20-55)

Table-2

Correlation of Extracellular matrix proteins (Collagen-1 and Fibronectin) with lung function and SA LP thickness.

Correlation parameters	NLFS	COPD-CS	COPD-ES
Collagen-1 in LP area vs lung function (FEV$_1$/FVC)	-	r'= -0.64 p<0.05	r'=0.35 p- ns
Fibronectin in LP area vs lung function (FEV$_1$/FVC)	-	r'=0.35 p- ns	r'=0.007 p- ns
Collagen-1 in LP area vs airway wall LP thickness (microns)	r'=0.05 p- ns	r'=0.59 p<0.05	r'=0.37 p<0.05
Fibronectin in LP area vs airway wall LP thickness (microns)	r'=0.4 p- ns	r'=-0.01 p- ns	r'=-0.01 p- ns
r'- Spearman r, p – p-value FEV1/FVC %- forced expiration / forced vital capacity%; FEF 25-75%- forced expiratory flow at 25-75% post bronchodilator.			

Figure 1: Representative images of the full airway wall thickness, with the αSMA+ive myofibroblast population marked with arrows, within the LP in the small airway wall of a) NC and b) COPD (45x magnification). An increase in thickness was observed in the clinical (smoker/COPD) groups in the c) LP, d) adventitia and e) smooth muscle layer.

Figure 2: Correlation between airway wall thickness and lung function indices a) %FEV1/FVC and b) FEF25-75% (more specific for small airways) in the combined COPD groups.

Figure 3: Representative images of αSMA+ive cells in the airway wall of a) NC, smoker (NLFS) and COPD current and ex-smokers. Increase in small airway αSMA+ive cells were observed in all three sub-layers of the sub-epithelial areas of the small airway wall, i.e. b) Rbm, c) LP and d) adventitia.

Figure 4: Representative images of collagen-1 and fibronectin deposition by % area in the airway wall of a) NC, smoker (NLFS) and COPD current and ex-smokers, with increases in percentage collagen-1 and fibronectin expression in pathological groups in both the (b, c) LP and (d, e) adventitia, with both current smoking and COPD effects.

Figure 5: Correlations between αSMA+ive myofibroblasts in the (a, c) Rbm and (b, d) LP of the three smoking/COPD groups (NLFS, COPD-CS, and ES) and indices of airflow, done independently: (a, b) %FEV$_1$/FVC and (c, d) FEF25-75%; (e) correlation between number of myofibroblast and thickness of LP among the combined pathological groups.
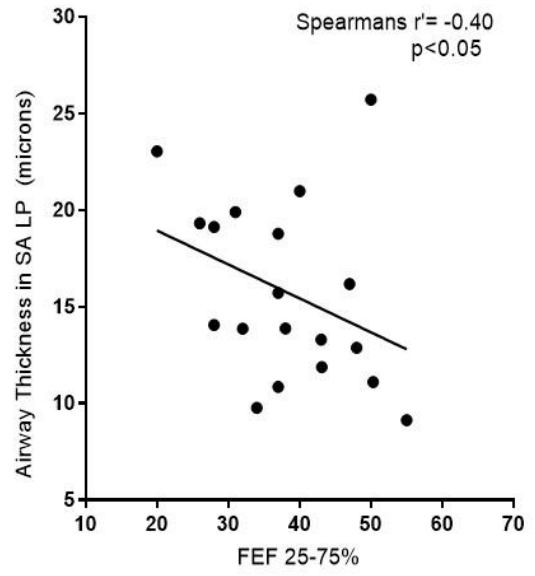
Figure 6: Correlations between the EMT marker S100A4 expression in the a) basal epithelial cells and b) Rbm, with αSMA+ive cells within both small airway wall and the Rbm; and between the two EMT markers c) S100A4 and d) Vimentin expressed in basal epithelial cells and LP thickening.
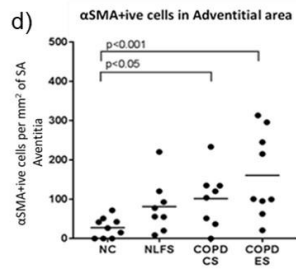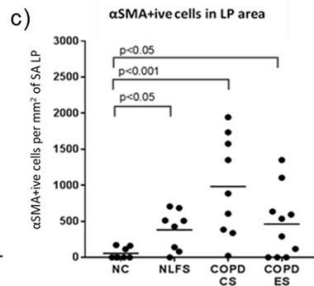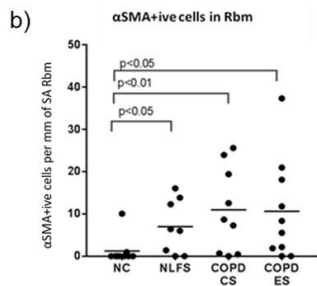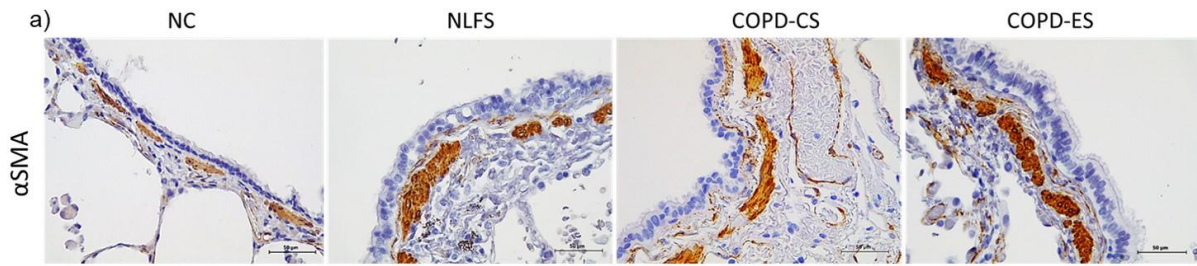
a)

LP thickness
SM thickness
Adventitia thickness

b)

LP Thickness
SM Thickness
Adventitia Thickness

50 μm

c) Lamina Propria (LP)

Average thickness (microns)

p<0.001
p<0.001
p<0.001

NC   NLFS   COPD   COPD
                ES   CS

d) Adventitia

Average thickness (microns)

p<0.01
p<0.01
p<0.001

NC   NLFS   COPD   COPD
                CS   ES

e) Smooth Muscle Layer

Average thickness (microns)

p<0.01
p<0.01
p<0.01

NC   NLFS   COPD   COPD
                CS   ES
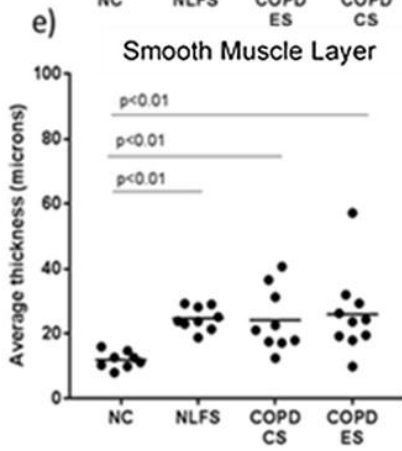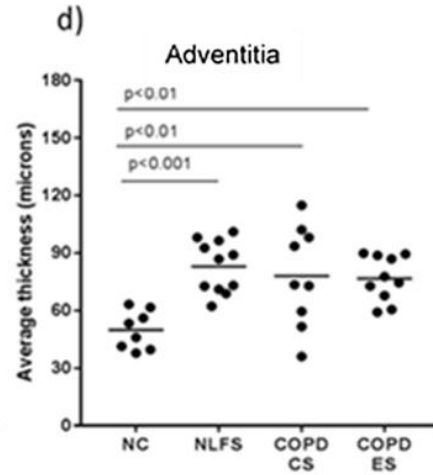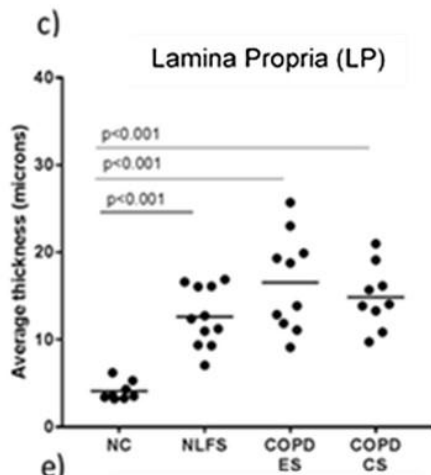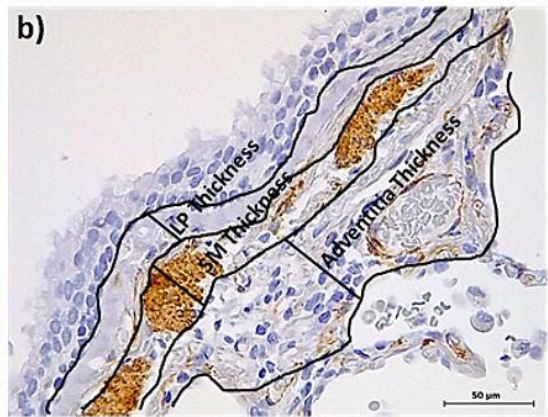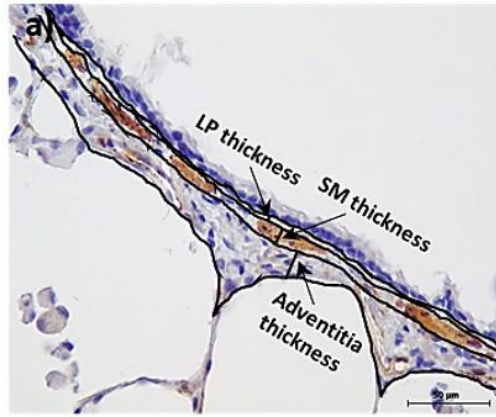
a) **LP thickness Vs. Lung Function**

Spearmans r'= -0.41
p<0.05

Airway wall thickness in LP (microns)

FEV$_1$/FVC
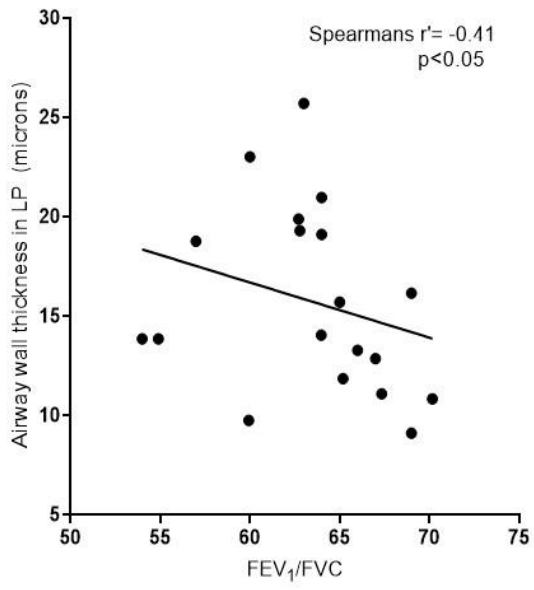
b) **LP thickness Vs. SA caliber**

Spearmans r'= -0.40
p<0.05

Airway Thickness in SA LP (microns)

FEF 25-75%

a)

| NC | NLFS | COPD-CS | COPD-ES |

αSMA

b) αSMA+ive cells in Rbm

c) αSMA+ive cells in LP area

d) αSMA+ive cells in Adventitial area

a)

| | NC | NLFS | COPD-CS | COPD-ES |

Collagen-1

Fibronectin

b) p<0.05
p<0.05

Percent Collagen-1 in SA LP

NC NLFS COPD CS COPD ES

c) p<0.05
p<0.001

Percent Collagen-1 in SA Advent

NC NLFS COPD CS COPD ES

d) p<0.05
p<0.001
p<0.01

Percent Fibronectin in SA LP

NC NLFS COPD CS COPD ES

e) p<0.05
p<0.001
p<0.01

Percent Fibronectin in SA Adventitia

NC NLFS COPD CS COPD ES

a) αSMA+ive cells in SA Rbm Vs Lung Function

▼ NLFS- r'=-0.11 p=0.38
● COPD-CS r'= -0.55 p=0.06
▲ COPD-ES r'=-0.42 p=0.11
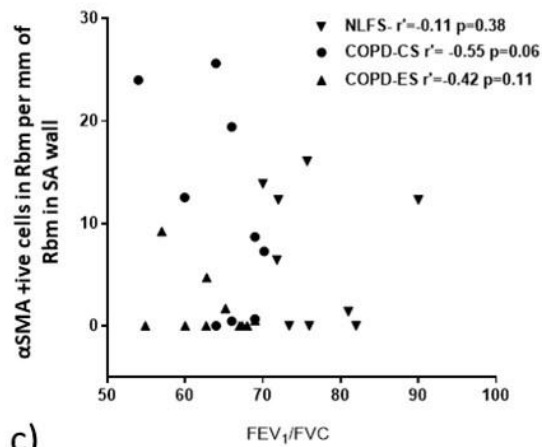
b) αSMA+ive cells in SA LP Vs Lung Function

▼ NLFS r'= -0.15 p= 0.38
● COPD-CS r'= -0.80 p<0.05
▲ COPD-ES r'=-0.61 p<0.05
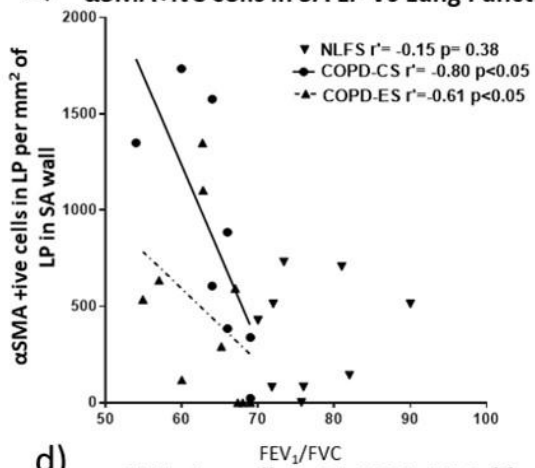
c) αSMA+ive cells in SA Rbm Vs SA Calibre

▼ NLFS r'=-0.08 p= 0.4135
● COPD-CS r'= -0.50 p=0.104
▲ COPD-ES r'=-0.102 p=0.39
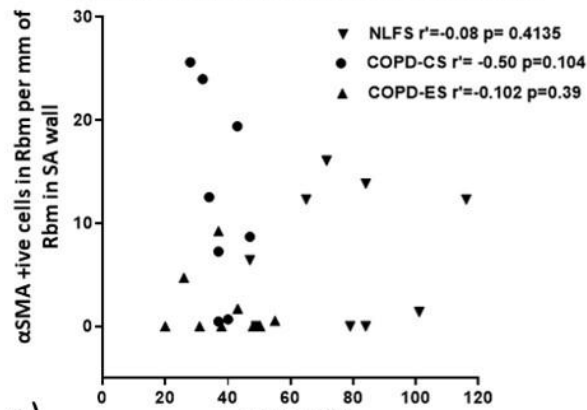
d) αSMA+ive cells in SA LP Vs SA Calibre

▼ NLFS r'= -0.15 p= 0.35
● COPD-CS r'= -0.57 p=0.07
-▲- COPD-ES r'=-0.68 p<0.05
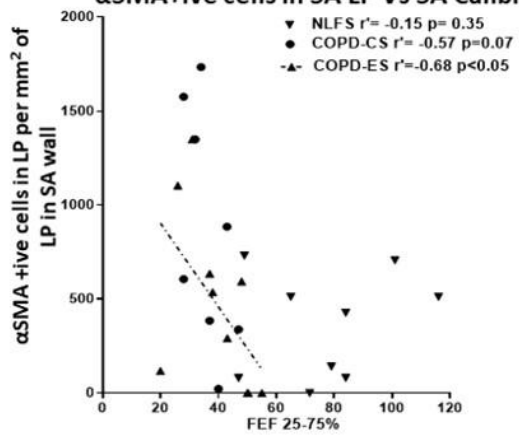
e) αSMA+ive cells in SA LP Vs LP thickness

Spearman r'= 0.43
p<0.05
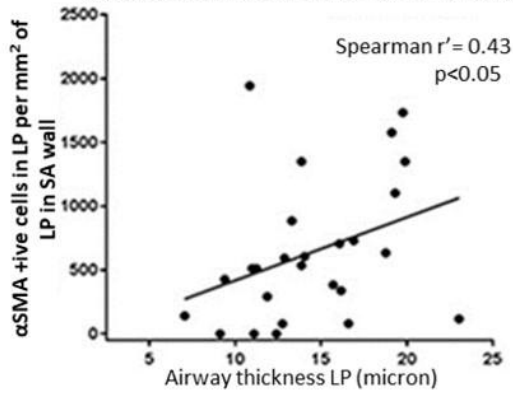
a) αSMA+ive Rbm cells vs S100A4+ive Epithelial cells

Spearman r'=0.46
p<0.05
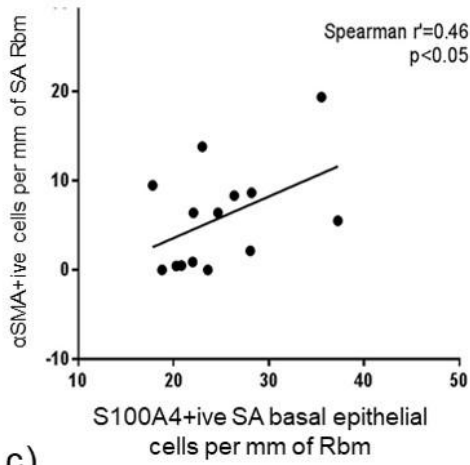
b) αSMA+ive Rbm cells vs S100A4+ive Rbm cells

Spearman r'=0.42
p=0.06
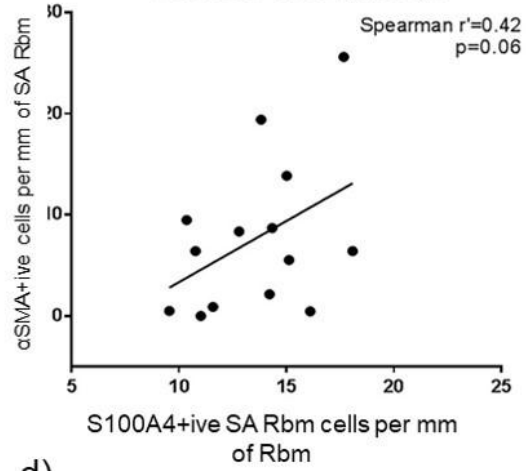
c) S100A4+ive basal cells vs SA wall LP thickness

Spearman r'=0.31
p<0.1
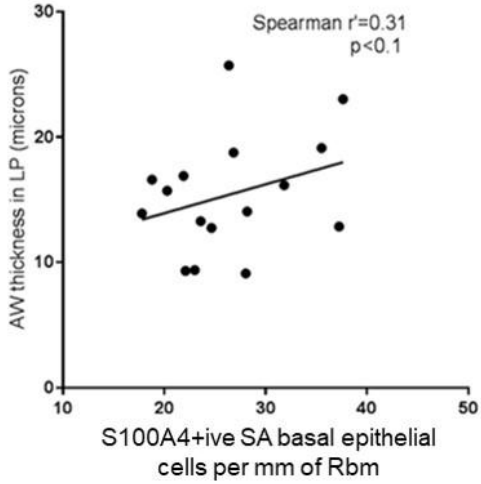
d) Vimentin+ive basal cells vs SA wall LP thickness
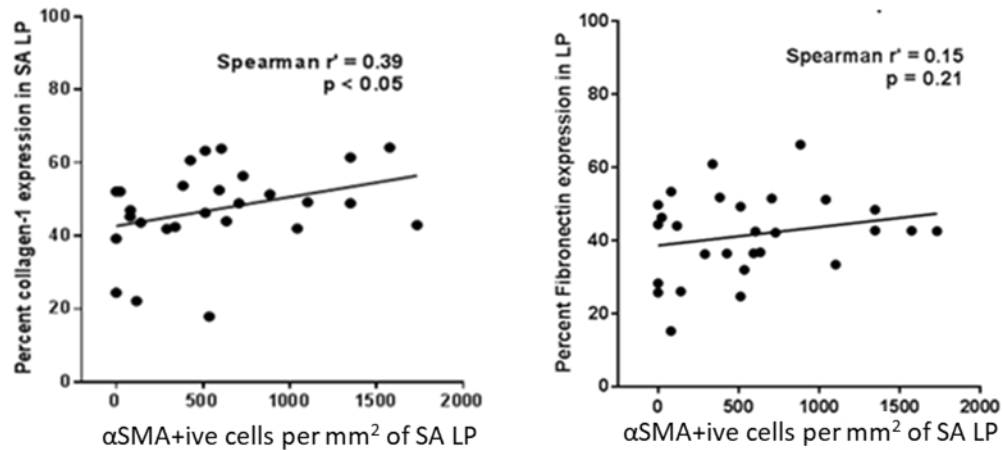
Spearman r'=0.47
p<0.05
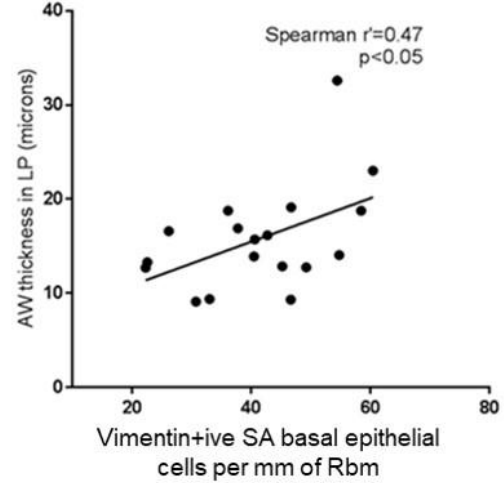
Supplement data and figure

Correlation of αSMA+ive cells to percent ECM changes

On evaluating the clinical groups, we found significant positive correlation between the number of αSMA+ive cells in LP with percent expression of collagen-1, but, not with fibronectin in the LP region (Supplementary Figure1 a, b)

Supplementary figure 1

Supplementary figure legend: Correlation analysis between airway αSMA+ive cell population in LP area and percent collagen-1 and fibronectin expression in LP (Figure 1 a, b) for all three clinical group (NLFS, COPD-CS, and COPD-ES).