




Increased myofibroblasts in the small airways, and relationship to remodelling and functional changes in smokers and COPD patients: potential role of epithelial–mesenchymal transition

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ABSTRACT

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

Methods: Lung resections from nonsmoker controls, normal lung function smokers and COPD current and ex-smokers were stained with anti-human α -smooth muscle actin (SMA), collagen 1 and fibronectin. α SMA⁺ cells were computed in reticular basement membrane (Rbm), lamina propria and adventitia and presented per mm of Rbm and mm² of lamina propria. Collagen-1 and fibronectin are presented as a percentage change from normal. All analyses including airway thickness were measured using Image-pro-plus 7.0.

Results: We found an increase in subepithelial lamina propria (especially) and adventitia thickness in all pathological groups compared to nonsmoker controls. Increases in α SMA⁺ myofibroblasts were observed in subepithelial Rbm, lamina propria and adventitia in both the smoker and COPD groups compared to nonsmoker controls. Furthermore, the increase in the myofibroblast population in the lamina propria was strongly associated with decrease in lung function, lamina propria thickening, increase in ECM protein deposition, and finally EMT activity in epithelial cells.

Conclusions: This is the first systematic characterisation of small airway myofibroblasts in COPD based on their localisation, 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.



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Myofibroblast populations increase in smokers and patients with COPD contributing to small airway fibrosis and obliteration. These changes might be driven by the process of epithelial to mesenchymal transition. <https://bit.ly/3lkouTU>

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Introduction

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) has defined COPD as a disease state characterised 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 pathological changes of COPD are observed in the airways, and functionally are mainly fibrosis and destruction in the small airways (SA) wall [2]. Emphysema of the peribronchial lung parenchyma occurs ~10 years after SA obstruction can be detected [3, 4], and predominantly in the 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, 7]. In the current study, we wished to further relate changes in a subpopulation of these stromal cells, namely myofibroblasts, to airway wall thickening and deposition of strategic representative extracellular matrix (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 strongly emphasised the relationship of these pathological small airway changes to appropriate measures of airflow obstruction [8].

Myofibroblasts are motile and contractile cells, with a high expression of α -smooth muscle actin (α SMA) protein. Previous studies in COPD evaluating expression of this protein marker for myofibroblasts in resected human airway tissue have been variable [9] in large and SA tissue. In contrast, findings from *in vitro* studies with fibroblasts isolated from the distal end of the airways from COPD patients did show increased contractile properties associated with increased myofibroblast numbers [10]. These findings suggest that myofibroblasts may be important in both large and small airways.

In the current study, we used anti- α SMA antibody immunohistochemistry to identify and quantify the SA wall myofibroblast population, taking care to dissociate them from smooth muscle cell bundles. In addition, we have descriptively analysed the localisation of these cells in the SA wall tissue sublayers. As mentioned, we have analysed 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-organisation of the ECM. Furthermore, we 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 epithelial–mesenchymal transition (EMT) activity that we have previously published as being related to airflow obstruction in COPD [11–14].

Materials and methods

Ethics approvals

The Tasmanian Health and Medical Human Research Ethics Committee approved the study (H0012374). Tissues from normal nonsmoker 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 SAs suitable for analysis (<2 mm internal diameter) from 40 patients were included (table 1). These non-normal subjects all had primary nonsmall cell lung cancer, with an approximately equal distribution of squamous and adenocarcinoma. 20 patients demonstrated mild–moderate GOLD stage I or II, of whom nine were COPD current smokers (CS) and 11 were COPD ex-smokers (ES) (>1 year smoking cessation). 11 individuals were normal lung function smokers (NLFS). Tissue from 10 normal controls (NC) was obtained from the tissue bank at the University of British

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TABLE 1 Patient demographics

	NC	NLFS	COPD-CS	COPD-ES
Subjects	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)
FEV₁/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)

Data are presented as n or median (range). NC: normal control; NLFS: normal lung function smoker; COPD-CS: COPD current smoker; COPD-ES: COPD ex-smoker; FEV₁: forced expiratory volume in 1 s; FVC: forced vital capacity; FEF_{25–75%}: forced expiratory flow at 25–75% of FVC; NS: nonsmokers; N/A: not available. [#]: post-bronchodilator values after 400 µg salbutamol. **: p<0.01 over NC (ordinary one-way ANOVA followed by Dunnett's multiple comparison test).

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 µm from individual paraffin-embedded blocks and immunostained for mouse monoclonal anti- α SMA (M0851, Dako; 1:400 dilution), mouse polyclonal anti-collagen-1 (AB34710, Abcam; 1:250 dilution) and polyclonal rabbit fibronectin (A0245, Dako; 1:1000 dilution) 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-labelled Envision (K4001; Dako) and diaminobenzidine (K3468; Dako). In addition, we incorporated an overlapping group of smoker and COPD tissues from a previous study [15] in which SAs had been stained in the same way as described earlier 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. The study included epithelium plus submucosal 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 the study of SA thickness, three to four SAs per patient were analysed, and of the total images, eight were selected using an online random number generator programme (www.stat trek.com). These tissue pictures were each divided into three subepithelial regions: the lamina propria (the area between the lower limit of the reticular basement membrane (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 1a 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 ones above and below. Based on tissue orientation, 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

α SMA⁺ cells with a fibroblast-like morphology were classified as myofibroblasts and enumerated in the SA wall Rbm, lamina propria and adventitia regions. Such α SMA⁺ 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 mm² of the respective area. For the expression of ECM “scar” proteins collagen-1 and fibronectin, diffuse brown staining was selected in the area of interest drawn manually for both lamina propria (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

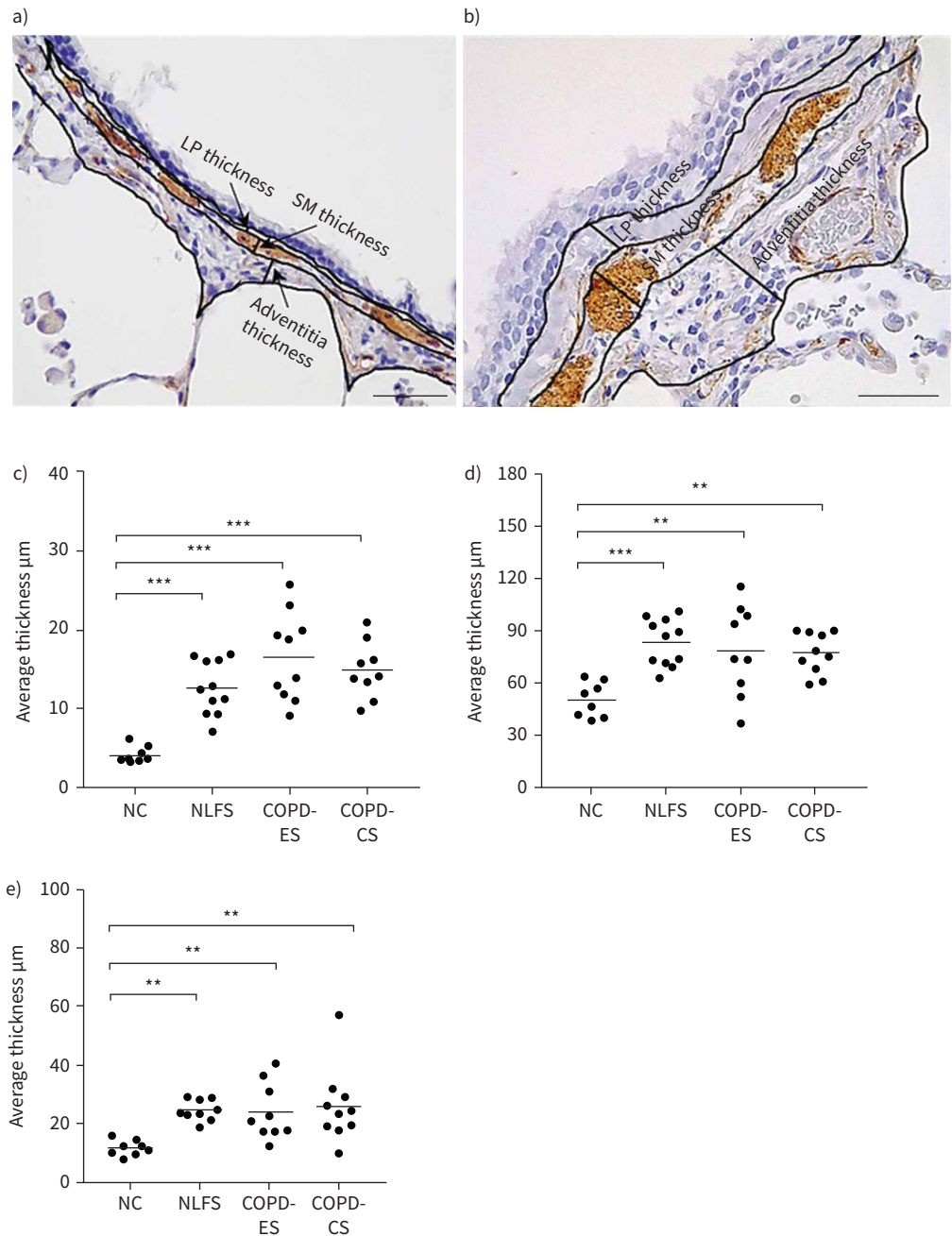


FIGURE 1 Representative images of the full airway wall thickness in the small airway wall of a) normal control (NC) and b) COPD. An increase in thickness was observed in the clinical (smoker/COPD) groups in the c) lamina propria (LP), d) adventitia and e) smooth muscle (SM) layer. NLFS: normal lung function smoker; COPD-CS: COPD current smoker; COPD-ES: COPD ex-smoker. Scale bars=50 μm . **: $p < 0.01$, ***: $p < 0.001$.

analysis software Image ProPlus 7.0. Furthermore, the software was used to generate a ratio of the collagen-1 and fibronectin staining in each selected area of interest, which is presented here as percentage 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. Nonparametric ANOVAs 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 completed 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 lamina propria and adventitia were significantly thicker in COPD subjects, with the lamina propria showing at least a 10-fold change overall compared to normal controls, whereas in the adventitial areas there was a two- to three-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, and almost equally in smokers, except in the lamina propria, 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 lamina propria subepithelial layer (figure 2a and b).

α SMA⁺ myofibroblasts in SA

Significant increases in SA α SMA⁺ 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 lamina propria and in actively smoking COPD. The increases in myofibroblast numbers in the SA lamina propria 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 percentage collagen-1 expression in the lamina propria (1.5-fold increase), the increase in the adventitia was much greater (five- to six-fold) in smokers and COPD patients compared to NC (figure 4a and c). This distribution difference was not seen for fibronectin, where the fold increase was more uniform (figure 4b and d).

Correlation of SA myofibroblasts with airflow physiological measures, and with SA wall lamina propria thickening

We present a complex set of correlations to show together the relationships between myofibroblast numbers in the Rbm and the lamina propria against airflow in the three clinical groups. Although group numbers are rather small, there was still a significant or near significant correlation between myofibroblast

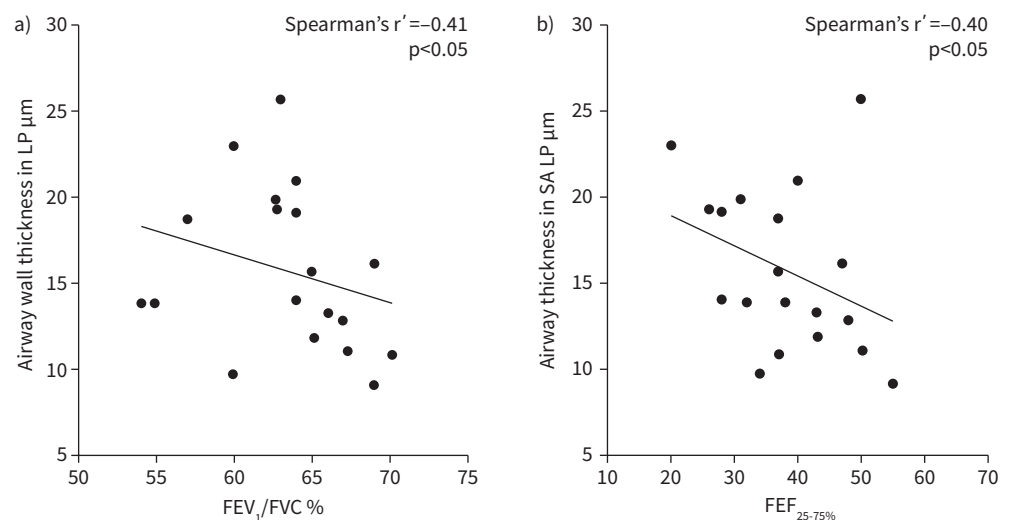


FIGURE 2 Correlation between airway wall thickness and lung function indices: a) forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) and b) forced expiratory flow at 25–75% of FVC ($\text{FEF}_{25-75\%}$) [more specific for small airways (SA)] in the combined COPD groups. LP: lamina propria.

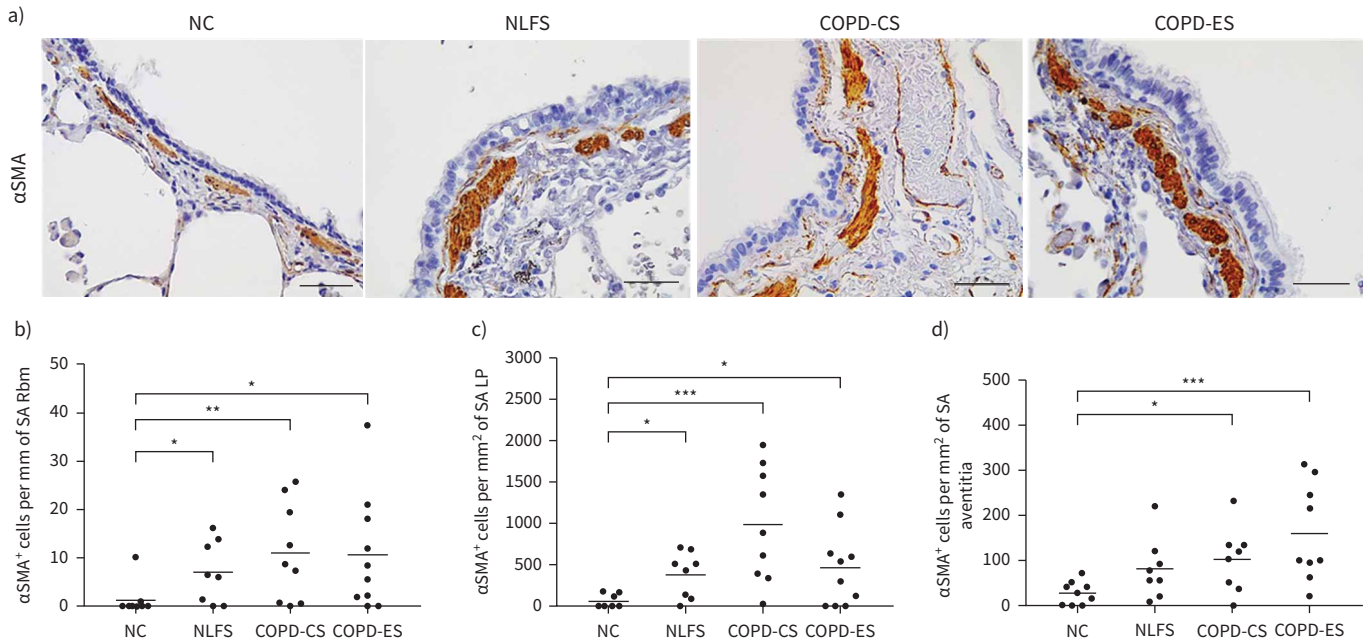


FIGURE 3 Representative images of α -smooth muscle actin [SMA]⁺ cells in the airway wall of a) normal control (NC), normal lung function smoker (NLFS) and COPD current (CS) and ex-smokers (ES). Increase in small airway (SA) α SMA⁺ cells were observed in all three sublayers of the subepithelial areas of the small airway wall: b) reticular basement membrane (Rbm); c) lamina propria (LP); and d) adventitia. Scale bars=50 μ m. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

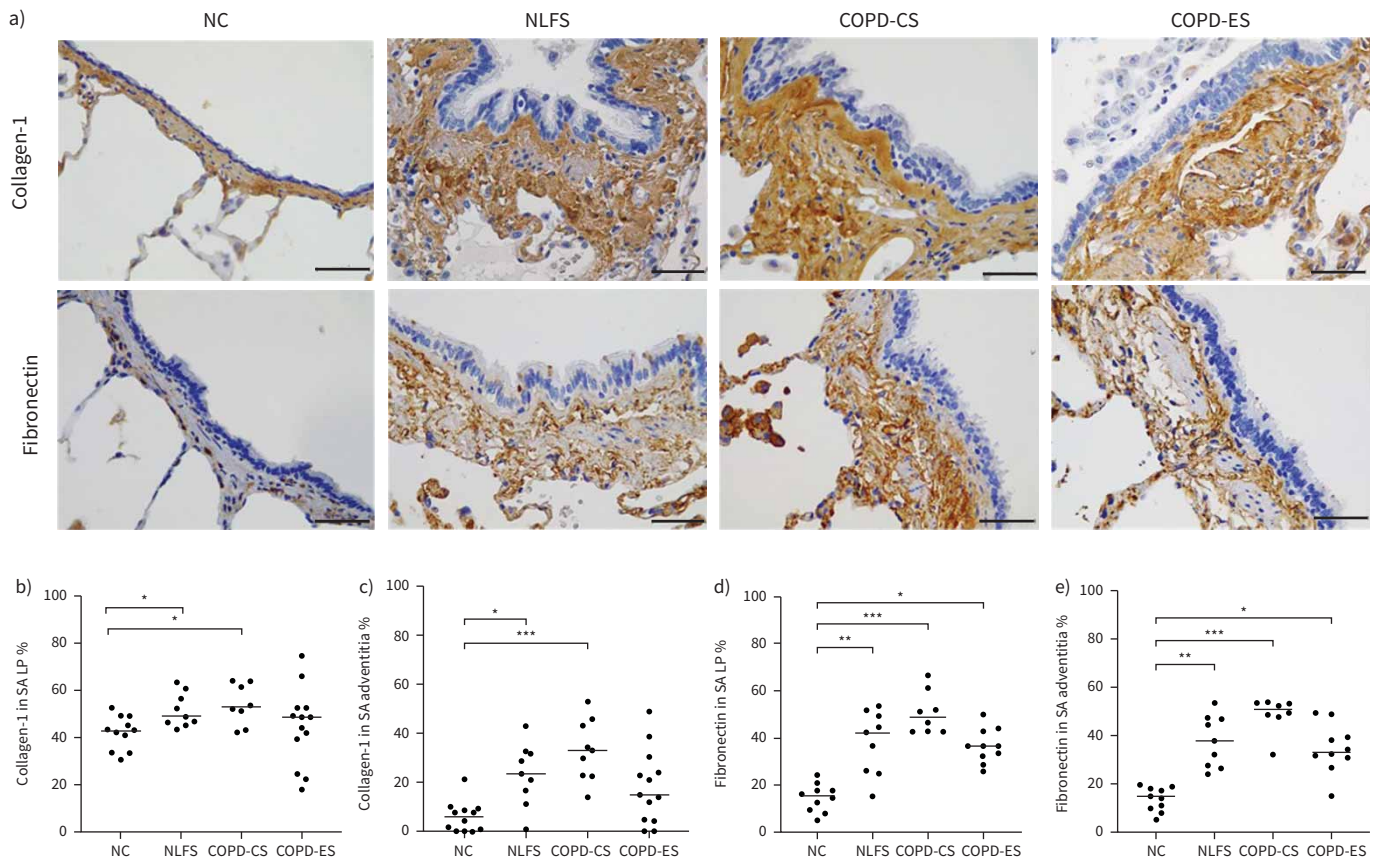


FIGURE 4 Representative images of collagen-1 and fibronectin deposition by percentage area in the airway wall of a) normal control (NC), normal lung function smoker (NLFS) and COPD current (CS) and ex-smokers (ES), with increases in percentage collagen-1 and fibronectin expression in pathological groups in both the b,d) lamina propria (LP) and c,e) adventitia, with both CS and COPD effects. SA: small airway. Scale bars=50 μ m. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

numbers *versus* decreases in airflow (obstruction), both as forced expiratory volume in 1 s (FEV₁)/forced vital capacity (FVC) ratio and forced expiratory flow at 25–75% of FVC, in both COPD groups, but not in the NLFS (figure 5a–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 lamina propria region and lamina propria thickness in all pathological groups (including current and ex-smokers) (figure 5e) showed a very similar picture, but suggesting both a smoking and COPD effect.

Correlation of percentage collagen-1 and fibronectin with lung function and SA wall lamina propria thickness in smoker/COPD groups

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

Correlation of EMT markers in SA wall cells versus number of α SMA⁺ myofibroblasts and airway thickening

EMT-marker expression in basal epithelial cells showed a positive association with α SMA⁺ myofibroblasts in Rbm (figure 6a and b) of the SA wall in combined COPD and NLFS groups with a ratio of ~4:1. Additionally, within the Rbm there was a relationship between S100A4 and α SMA⁺ cells with a ratio of ~1.5:1, suggesting a transition of mesenchymal-marker positive cells towards myofibroblasts, with α SMA cell expression thought to represent a late manifestation of the EMT process. Equally notably, there was a positive relationship between basal epithelial cell EMT activity, as indicated by both S100A4 and vimentin expression, and the (enhanced) thickness of the lamina propria.

Discussion

A core finding in this study was the increase in α SMA⁺ myofibroblasts in the SA wall of COPD patients and its association with a major increase in the lamina propria sublayer thickness. In addition, there were 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 to be secondary changes in the myofibroblast population and consequential lamina propria 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 have suggested previously [12, 14, 16, 17].

It has been accepted that one of the principal causes for airflow limitation in COPD is the airway wall tissue remodelling through re-organisation of the ECM [18]. In the current study, we analysed two important markers of ECM pathology, collagen-1 and fibronectin, both of which have been described as localised to areas with increased proliferation of myofibroblasts in COPD [19, 20]. 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 sublayers. Furthermore, 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 consequences of these anatomic variations will need further effort, but we found that collagen-1 but not fibronectin in lamina propria was associated with airflow obstruction in COPD. For fibronectin, its cellular immune-modulatory roles may be more important than structural ones [21].

Our current observation of an increase in collagen-1 is in agreement with the earlier studies by HARJU *et al.* [20] in SA tissues where an overall increase in both collagen-1 and -3 subtypes in GOLD stages I and II COPD were observed in the SA lamina propria. Both these studies contrast with observations by ANNONI *et al.* [22], 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 patients. Furthermore, ECM changes seemed to regress in COPD-ES, and indeed relationships to lung function were significant in current-smoking COPD patients, but not ex-smokers. The cause of such regression

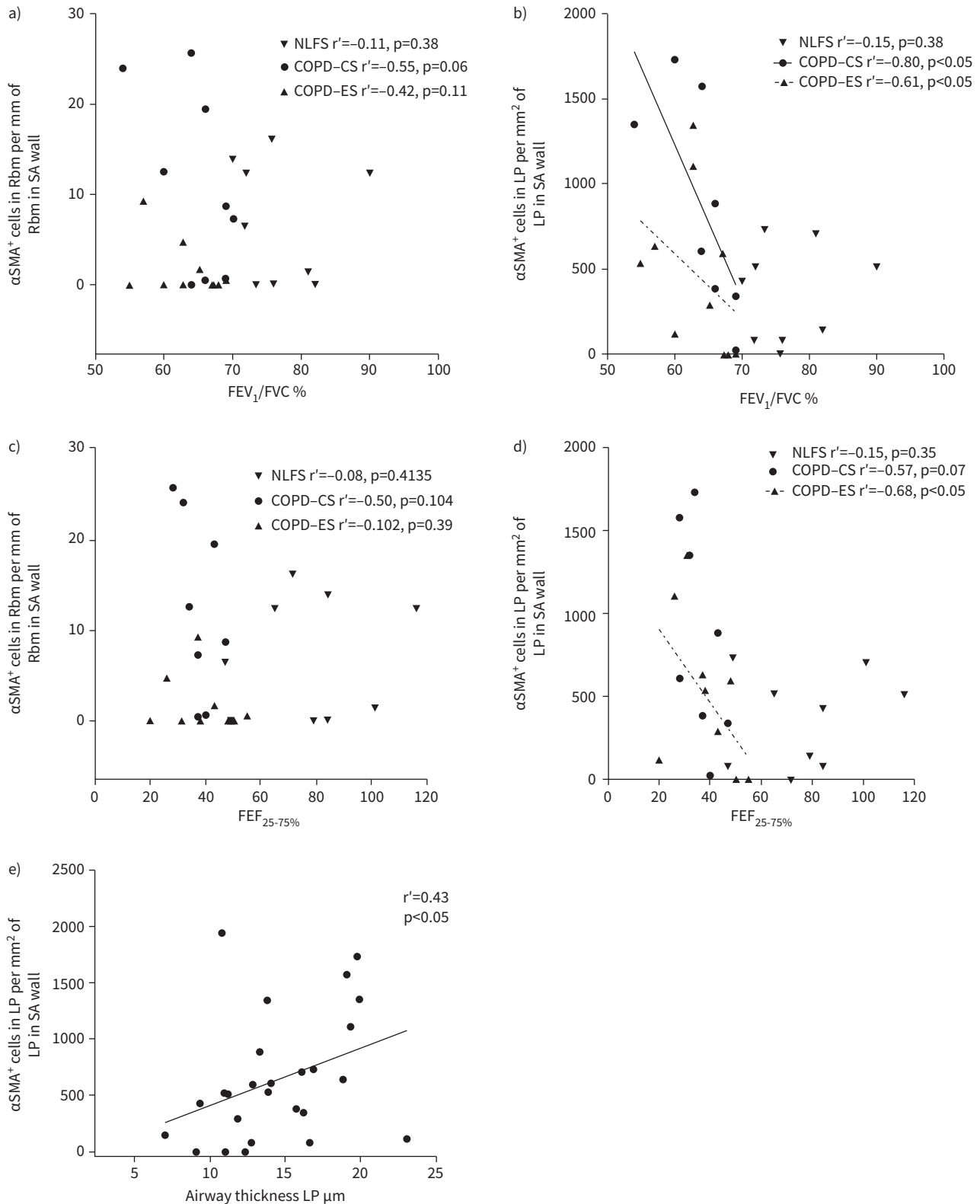


FIGURE 5 Correlations between α -smooth muscle actin (SMA)⁺ myofibroblasts in the a, c) reticular basement membrane (Rbm) and b, d) lamina propria (LP) of the three smoking/COPD groups (normal lung function smoker (NLFS) and COPD current (CS) and ex-smokers (ES)) and indices of airflow, done independently: a, b) forced expiratory volume in 1 s (FEV₁)/forced vital capacity (FVC) ratio and c, d) forced expiratory flow at 25–75% of FVC (FEF_{25–75%}); e) correlation between number of myofibroblasts and thickness of LP among the combined pathological groups. SA: small airway; r': Spearman's r.

TABLE 2 Correlation of extracellular matrix proteins (collagen-1 and fibronectin) with lung function and small airway lamina propria thickness

	NLFS	COPD-CS	COPD-ES
Collagen-1 in lamina propria area versus lung function (FEV₁/FVC)		r'=-0.64 p<0.05	r'=0.35 p-value NS
Fibronectin in lamina propria area versus lung function (FEV₁/FVC)		r'=0.35 p-value NS	r'=0.007 p-value NS
Collagen-1 in lamina propria area versus airway wall lamina propria thickness (μm)	r'=0.05 p-value NS	r'=0.59 p<0.05	r'=0.37 p<0.05
Fibronectin in lamina propria area versus airway wall lamina propria thickness (μm)	r'=0.4 p-value NS	r'=-0.01 p-value NS	r'=-0.01 p-value NS

NLFS: normal lung function smoker; COPD-CS: COPD current smoker; COPD-ES: COPD ex-smoker; FEV₁: forced expiratory volume in 1 s; FVC: forced vital capacity; r': Spearman's r; NS: nonsignificant.

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 α SMA microfilaments [23, 24]. Surprisingly, few reports have been published that our current

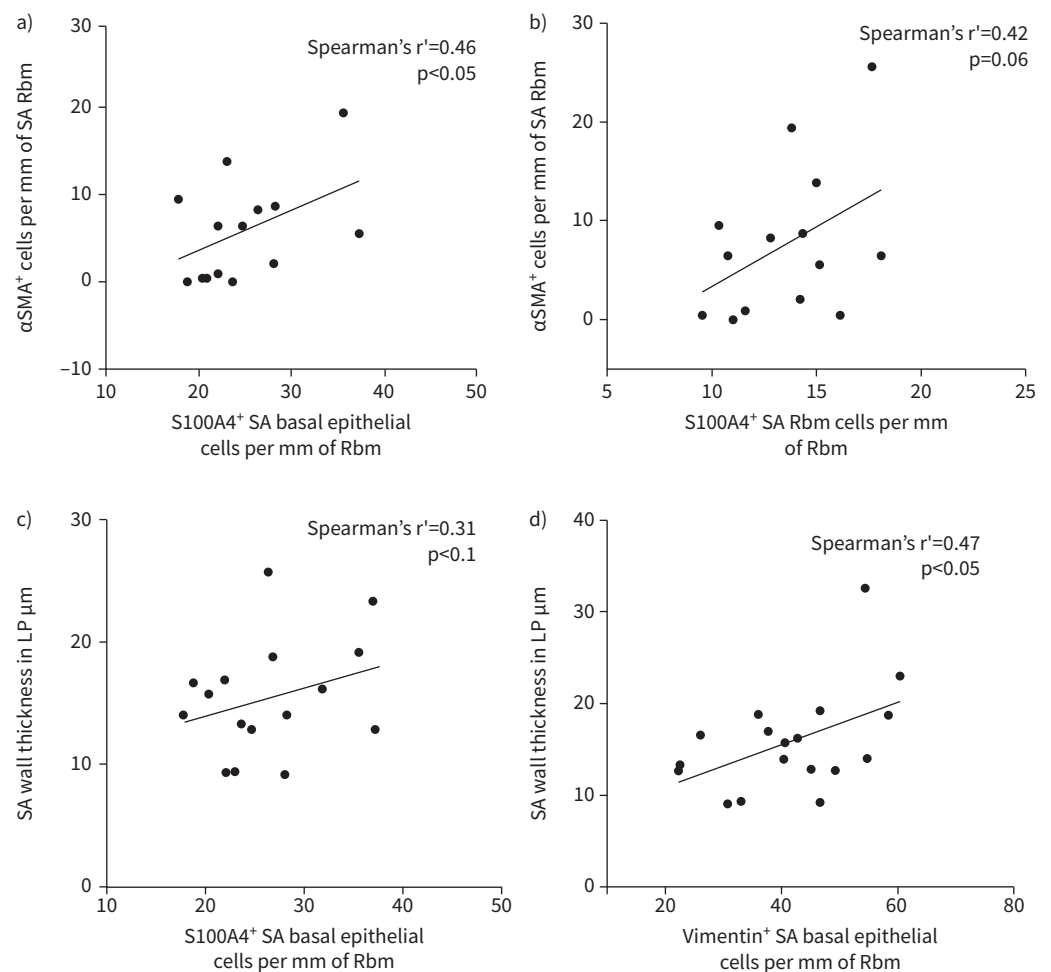


FIGURE 6 Correlations between the epithelial-mesenchymal transition (EMT) marker S100A4⁺ expression in the a) basal epithelial cells and b) reticular basement membrane (Rbm), with α -smooth muscle actin (SMA)⁺ cells within both small airway (SA) wall and the Rbm; and between the two EMT markers c) S100A4 and d) vimentin expressed in basal epithelial cells and lamina propria (LP) thickening.

data could be compared to. Our finding of an increase in α SMA⁺ myofibroblasts (on morphology criteria) is in contrast to that of KARVONEN *et al.* [25], who showed a decrease in expression α SMA⁺ cells in the bronchioles of COPD patients compared to nonsmoker controls. The differences in the findings are probably due to the counting strategy and area under consideration. Thus, while KARVONEN *et al.* counted α SMA⁺ cells in the whole of the subepithelial wall, in the current study we took each sublayer in turn. Indeed, KARVONEN *et al.* considered the Rbm and the muscle layer as part of the lamina propria for SA. However, our findings are similar to those of HARJU *et al.* [20], who provided a descriptive analysis of the SA tissue and evidence of colocalisation of α SMA⁺ cells with collagen subtypes as well as mesenchymal markers such as vimentin in the SA wall. Like us, they suggested that α SMA⁺ myofibroblasts could be responsible for the increased accumulation of collagen-1 and fibronectin in the SA lamina propria of COPD patients, and, like us, others have suggested that myofibroblasts are responsible for airway wall thickening secondary to ECM accumulation (supplementary figure S1) [20].

We have taken these matters substantially further than previous reports, observing physiological measures of reduced airway calibre with increasing myofibroblast density accompanying changes in ECM, and suggesting possible involvement of this cell type in SA remodelling and narrowing and ultimately obliteration [2, 26]. Therefore, we should emphasise 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 [15] suggested that the underlying mechanism for airway wall remodelling is through the induction of EMT [11, 27], as part of a broader epithelial-gene reprogramming [28, 29]. 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 [11, 15]. Transforming growth factor (TGF)- β 1 pathways are likely to contribute to driving EMT in COPD, *via* nuclear transcription factors such as pSMAD2/3 with reduction in the inhibitory SMAD7 [13, 30]. Interestingly, TGF- β 1 pathways are also suggested to play a crucial role in the development of myofibroblasts from tissue fibroblasts through activation of the SMAD pathway [26, 31], so although we believe EMT to be a key mechanism in COPD pathogenesis [32], it is unlikely to be the only growth factor driven mechanism operating throughout the whole thickness of the SA wall [33]. In addition to the TGF- β 1 pathway, we and others have previously shown 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 [28, 34, 35].

There are limitations to our study. The numbers of individual subjects contributing tissue samples per clinical group were relatively small due to study logistics, but even so many of the findings are statistically robust for the most part, and consistently so, emphasising the strength of the signals obtained. Furthermore, this study 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 the possible contribution of age in addition to smoking and disease. We did find that smokers with normal lung function had thick airway walls, increased myofibroblast numbers and significantly higher ECM changes compared to normal subjects and closer to levels of patients with COPD [36]. 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 ageing was observed in normal subjects, especially between the fourth and sixth generation airways, which were indeed thicker in patients with COPD [37–39]. Clinical features such as air trapping seems to be common between ageing lungs and COPD. Another generic problem in this method of tissue research is that the COPD-CS 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.* [8] 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 are still 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 [40].

Conflict of interest: M.S. Eapen has nothing to disclose. W. Lu has nothing to disclose. T.L. Hackett has nothing to disclose. G.K. Singhera has nothing to disclose. M.Q. Mahmood has nothing to disclose. A. Hardikar has nothing to disclose. C. Ward has nothing to disclose. E.H. Walters has nothing to disclose. S.S. Sohal reports personal fees from Chiesi outside the submitted work.

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References

- 1 Vestbo J, Hurd SS, Agustí AG, *et al.* Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2013; 187: 347–365.
- 2 Koo HK, Vasilescu DM, Booth S, *et al.* Small airways disease in mild and moderate chronic obstructive pulmonary disease: a cross-sectional study. *Lancet Respir Med* 2018; 6: 591–602.
- 3 Thurlbeck WM, Dunnill MS, Hartung W, *et al.* A comparison of three methods of measuring emphysema. *Human Pathol* 1970; 1: 215–226.
- 4 Eapen MS, Myers S, Walters EH, *et al.* Airway inflammation in chronic obstructive pulmonary disease (COPD): a true paradox. *Expert Rev Respir Med* 2017; 11: 827–839.
- 5 O'Donnell DE, Laveneziana P. Physiology and consequences of lung hyperinflation in COPD. *Eur Respir Rev* 2006; 15: 61–67.
- 6 Eapen MS, McAlinden K, Tan D, *et al.* Profiling cellular and inflammatory changes in the airway wall of mild to moderate COPD. *Respirology* 2017; 22: 1125–1132.
- 7 Eapen MS, Hansbro PM, McAlinden K, *et al.* Abnormal M1/M2 macrophage phenotype profiles in the small airway wall and lumen in smokers and chronic obstructive pulmonary disease. *Sci Rep* 2017; 7: 13392.
- 8 Hogg JC, Paré PD, Hackett TL. The contribution of small airway obstruction to the pathogenesis of chronic obstructive pulmonary disease. *Physiol Rev* 2017; 97: 529–552.
- 9 Löfdahl M, Kaarteenaho R, Lappi-Blanco E, *et al.* Tenascin-C and alpha-smooth muscle actin positive cells are increased in the large airways in patients with COPD. *Respir Res* 2011; 12: 48.
- 10 Hallgren O, Rolandsson S, Andersson-Sjöland A, *et al.* Enhanced ROCK1 dependent contractility in fibroblast from chronic obstructive pulmonary disease patients. *J Transl Med* 2012; 10: 171.
- 11 Sohal SS, Reid D, Soltani A, *et al.* 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.
- 12 Sohal SS, Reid D, Soltani A, *et al.* Evaluation of epithelial mesenchymal transition in patients with chronic obstructive pulmonary disease. *Respir Res* 2011; 12: 130.
- 13 Sohal SS, Soltani A, Reid D, *et al.* A randomised 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. *Int J Chron Obstruct Pulmon Dis* 2014; 9: 533–542.
- 14 Sohal SS, Walters EH. Role of epithelial mesenchymal transition (EMT) in chronic obstructive pulmonary disease (COPD). *Respir Res* 2013; 14: 120.
- 15 Mahmood MQ, Sohal SS, Shukla SD, *et al.* Epithelial mesenchymal transition in smokers: large versus small airways and relation to airflow obstruction. *Int J Chron Obstruct Pulmon Dis* 2015; 10: 1515–1524.
- 16 Sohal SS, Eapen MS, Ward C, *et al.* Epithelial-mesenchymal transition: a necessary new therapeutic target in chronic obstructive pulmonary disease? *Am J Respir Crit Care Med* 2017; 196: 393–394.
- 17 Sohal SS, Eapen MS, Ward C, *et al.* Airway inflammation and inhaled corticosteroids in COPD. *Eur Respir J* 2017; 49: 1700289.
- 18 Ito JT, Lourenço JD, Righetti RF, *et al.* Extracellular matrix component remodeling in respiratory diseases: what has been found in clinical and experimental studies? *Cells* 2019; 8: 342.
- 19 Stylianou P, Clark K, Gooptu B, *et al.* Tensin1 expression and function in chronic obstructive pulmonary disease. *Sci Rep* 2019; 9: 18942.
- 20 Harju T, Kinnula VL, Pääkkö P, *et al.* Variability in the precursor proteins of collagen I and III in different stages of COPD. *Respir Res* 2010; 11: 165.
- 21 Thomas AH, Edelman ER, Stultz CM. Collagen fragments modulate innate immunity. *Exp Biol Med* 2007; 232: 406–411.
- 22 Annoni R, Lanças T, Yukimatsu Tanigawa R, *et al.* Extracellular matrix composition in COPD. *Eur Respir J* 2012; 40: 1362–1373.
- 23 Murray LA, Knight DA, Laurent GJ. Fibroblasts. In: Barnes PJ, Drazen JM, Rennard SI, *et al.*, eds. *Asthma and COPD*. 2nd Edn. Oxford, Academic Press, 2009; 193–200.
- 24 Pellegrin S, Mellor H. Actin stress fibres. *J Cell Sci* 2007; 120: 3491–3499.
- 25 Karvonen HM, Lehtonen ST, Harju T, *et al.* Myofibroblast expression in airways and alveoli is affected by smoking and COPD. *Respir Res* 2013; 14: 84.
- 26 Gu L, Zhu YJ, Yang X, *et al.* Effect of TGF- β /Smad signaling pathway on lung myofibroblast differentiation. *Acta Pharm Sin* 2007; 28: 382–391.
- 27 Crystal RG. Airway basal cells. The “smoking gun” of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2014; 190: 1355–1362.
- 28 Mahmood MQ, Walters EH, Shukla SD, *et al.* β -catenin, Twist and Snail: transcriptional regulation of EMT in smokers and COPD, and relation to airflow obstruction. *Sci Rep* 2017; 7: 10832.
- 29 Sohal SS. Airway basal cell reprogramming and epithelial-mesenchymal transition: a potential key to understanding early chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2018; 197: 1644–1645.

- 30 Mahmood MQ, Reid D, Ward C, *et al.* Transforming growth factor (TGF) β_1 and Smad signalling pathways: a likely key to EMT-associated COPD pathogenesis. *Respirology* 2017; 22: 133–140.
- 31 Harris WT, Kelly DR, Zhou Y, *et al.* Myofibroblast differentiation and enhanced TGF-B signaling in cystic fibrosis lung disease. *PLoS One* 2013; 8: e70196.
- 32 Eapen MS, Sohal SS. Update on the pathogenesis of COPD. *N Engl J Med* 2019; 381: 2483–2484.
- 33 Liu G, Philp AM, Corte T, *et al.* Therapeutic targets in lung tissue remodelling and fibrosis. *Pharmacol Ther* 2021; 225: 107839.
- 34 Eapen MS, Sohal SS. WNT/ β -catenin pathway: a novel therapeutic target for attenuating airway remodelling and EMT in COPD. *EBioMedicine* 2020; 62: 103095.
- 35 Carlier FM, Dupasquier S, Ambroise J, *et al.* Canonical WNT pathway is activated in the airway epithelium in chronic obstructive pulmonary disease. *EBioMedicine* 2020; 61: 103034.
- 36 Telenga ED, Oudkerk M, van Ooijen PM, *et al.* Airway wall thickness on HRCT scans decreases with age and increases with smoking. *BMC Pulm Med* 2017; 17: 27.
- 37 Su ZQ, Guan WJ, Li SY, *et al.* Evaluation of the normal airway morphology using optical coherence tomography. *Chest* 2019; 156: 915–925.
- 38 Bommart S, Marin G, Bourdin A, *et al.* Computed tomography quantification of airway remodelling in normal ageing subjects: a cross-sectional study. *Eur Respir J* 2015; 45: 1167–1170.
- 39 Occhipinti M, Larici AR, Bonomo L, *et al.* Aging airways: between normal and disease. A multidimensional diagnostic approach by combining clinical, functional, and imaging data. *Aging Dis* 2017; 8: 471–485.
- 40 Lu W, Sharma P, Eapen MS, *et al.* Inhaled corticosteroids attenuate epithelial mesenchymal transition: implications for COPD and lung cancer prophylaxis. *Eur Respir J* 2019; 54: 1900778.