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Epithelial-mesenchymal transition (EMT) changes in non-small cell lung cancer patients with early COPD

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Running Head: EMT activities in COPD implicate high lung cancer prevalence.

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Abstract

Background: EMT might be central to lung cancer development in smokers and COPD. We illustrate EMT changes in a broader demographic of patient groups who were diagnosed with non-small cell lung cancer (adenocarcinoma and squamous cell carcinoma). These included COPD current and ex-smokers, patients with small airway (SA) disease and normal lung function smokers compared to normal controls (NC).

Methods: We had access to surgically resected SA tissue from 46 subjects and assessed for airway wall thickness and immunohistochemically for EMT biomarkers: E-cadherin, N-cadherin, S100A4, Vimentin, and epidermal growth factor receptor (EGFR). All tissue analysis was done with a computer and microscope-assisted Image-pro Plus 7.0 software.

Results: Airway wall thickness significantly increased across all pathological groups (p<0.05) compared to NC. SA epithelial E-cadherin expression markedly decreased (p<0.01), and increase in N-cadherin, Vimentin, S100A4, and EGFR expression was observed in all pathological groups compared to NC (p<0.01). Vimentin-positive cells in reticular basement membrane (Rbm), lamina propria (LP), and adventitia showed a similar trend to epithelium across all pathological groups (p<0.05); however, such changes were only observed in Rbm for S100A4 (p<0.05). Vimentin was higher in adenocarcinoma versus squamous cell carcinoma; in contrast, S100A4 was higher in the squamous cell carcinoma group. EGFR and N-cadherin expressions in both phenotypes were markedly higher than E-cadherin, Vimentin and S100A4 (p<0.0001).

Conclusion: EMT is an active process in the SA of smokers and COPD diagnosed with non-small cell lung cancer, contributing to small airway remodelling and cancer development as seen in these patients.

Keywords: EMT, COPD, small airway, NSCLC, EGFR
Introduction

Over 3 million people died from Chronic obstructive pulmonary disease (COPD) in 2019, which is currently the third most common cause of death globally [1]. The main feature of COPD includes a gradual narrowing of the airways, small airway fibrosis and obliteration, resulting in persistent airflow limitation and difficulty breathing when performing physical activities [2]. On the other hand, lung cancer is the primary cause of cancer-related death, and there were over 2 million newly diagnosed cases worldwide in 2020 [3]. About 80-85% of lung cancer is non-small cell lung cancer (NSCLC), with the highest number of cancer-related deaths globally. Adenocarcinoma is the most frequently occurring histological type of NSCLC, accounting for 40% of cases, followed by squamous cell carcinoma, which makes up approximately 25-30% of cases [4, 5]. Studies have shown that COPD associated with smoking and the presence of emphysema in particular is a risk factor with a four- to six-fold more significant risk of developing lung cancer than smokers with normal lung function [6, 7]. Lung cancer is a common cause of death in COPD patients, especially those with severe disease [8, 9]. COPD and lung cancer have common characteristics, including elevated mortality rates and risk factors such as cigarette smoking. Several mechanisms have been elucidated for the correlation between COPD and lung cancer, such as genetic mutations, chronic inflammation, and dysregulated activation of bronchioalveolar stem cells but especially epithelial to mesenchymal transition (EMT) [10, 11].

EMT is a process of epithelial cells undergoing multiple molecular changes to obtain a mesenchymal phenotype with the potential to migrate through the reticular basement membrane (Rbm) to the subepithelial lamina propria [12, 13]. EMT is a widely recognised mechanism in embryonic development (Type-1 EMT), pathological forms promote fibrosis (Type-2 EMT) and epithelial malignancy (Type-3 EMT), mostly seen in cancer development, invasion, and metastasis [14, 15]. Furthermore, we have reported EMT activity as central to fibrotic small airway remodelling and epithelial malignancies in COPD patients [11, 16-18]. Hence, EMT may play a vital role in the connection between COPD and lung cancer, which can be triggered by smoking.

To explore this further, we investigate here, EMT-related changes and airway wall thickness in the small airways of broader demographic patient groups who are smokers with lung cancer and with or without COPD. We also made an attempt to differentiate EMT activity based on the type of lung cancer in these patients.
Materials and Methodology

Participants

We had access to surgically resected lung tissues, away from the primary tumour mass, from thirty-five participants who consented at Royal Hobart Hospital for the clinical samples (Table 1). The Thoracic Surgeon, Ashutosh Hardikar (co-author), performed the surgeries according to appropriate guidelines. There were twenty-two patients with adenocarcinoma and thirteen patients with squamous cell carcinoma. Nineteen participants had demonstrated mild-moderate Global Initiative for Obstructive Lung Disease (GOLD) stage I and II COPD, of which nine were current smokers with COPD (COPD-CS), and ten were ex-smokers (> 1 year smoking cessation) with COPD (COPD-ES). In addition, seven individuals were normal lung function smokers (NLFS), and nine individuals had small airway disease (SAD). The Tasmanian Health & Medical Human Research Ethics Committee approved the study (Ethics ID: H0012374), while tissues from eleven healthy non-smoking individuals who had passed away due to causes unrelated to pulmonary diseases were obtained from the James Hogg Lung Registry at the University of British Columbia (Ethics ID: H00–50110).

Immunohistochemistry

Resected small airway tissue sections were cut at 3μm from the paraffin-embedded blocks as we previously reported [19-21]. Tissue sections were dewaxed and rehydrated in distilled water following the order with xylene, absolute ethanol and 70% ethanol (v/v) in distilled water, respectively. Heat-Induced Epitope Retrieval was used with a Decloaking Chamber™ (Biocare Medical, Queensland, Australia) at 110 °C for 15 minutes with the heat retrieval citrate solution (pH6, S2369, Dako, Victoria, Australia). Endogenous enzyme blocking was performed with 3% hydrogen peroxide in distilled water (v/v). Immunohistochemical staining was carried out with epithelial junctional marker E-cadherin (1:50 dilution, M3612, Dako, Victoria, Australia) and mesenchymal markers mouse monoclonal N-cadherin (1:100 dilution, ab98952, Abcam, Victoria, Australia), rabbit polyclonal S100A4 (1:1000 dilution, A5114, Dako, Victoria, Australia), mouse monoclonal Vimentin (1:200 dilution, M7020 Dako, Victoria, Australia), and the epidermal growth factor receptor (EGFR, 1:150 dilution, ab32077, Abcam, Victoria, Australia), followed by an enzyme-conjugated polymer backbone that carries secondary antibodies (Dako REAL EnVision detection system, K5007, Dako, Victoria, Australia), with 3,3’-Diaminobenzidine (DAB+) as the chromogen for visualisation. In addition, for correlation purposes, we incorporated an overlapping group of smokers and COPD tissues from a previous study [16] in which SAs had been stained in the same way for alpha-smooth muscle actin (α-SMA), collagen-1 and Fibronectin.
Quantification of biomarkers expression in small airway

All quantification was done by using a Leica camera (ICC50W, Leica, NSW, Australia), featured a microscope (DM500, Leica, NSW, Australia), and computer-assisted Image-pro Plus 7.0 (Media Cybernetics, Maryland, U.S.) software. Non-overlapping images of small airways in each tissue section were taken at a 40X bright field. Eight images were randomly selected from the total number of images using an online random number generator program (www.calculatorsoup.com) for quantification. The number of cells positive for S100A4 and Vimentin in small airway epithelium and Rbm were quantified and normalised per millimetre of Rbm length. Additionally, the epithelial percentage positive expression of EGFR and EMT markers (N-cadherin, E-Cadherin, S100A4 and Vimentin) was measured. The number of positive cells in lamina propria (LP) and adventitia were presented as cells per mm² of the area, respectively.

Measurement of small airway wall thickness

The small airway images of each subject were taken at 40X bright field, and eight of the total images were randomly selected (same as mentioned above). The airway sub-epithelial regions were divided into: the Rbm, which refers to the area between the lower margin of the epithelium and the upper margin of the LP; the LP presents the area between the lower limit of the Rbm and the upper margin of the muscle layer; the adventitia, is the region between the lower margin of the muscle layer and the margin of the alveolar tissue interface. The thickness of each layer was determined using Image-pro Plus 7.0 software by drawing a line along the outer margins of each layer and using the software's automated distance and area calculator to calculate the distance and area.

Statistics

GraphPad (La Jolla, CA, U.S.) version 9.0 was used for statistical analysis. Non-parametric analyses of variance were performed using the Kruskal-Wallis Test; specific group differences without correction for multiple comparisons were assessed using a one-way ANOVA test with Dunn's multiple comparison test. Correlation analysis was performed with regression analyses using Spearman's rank test. The statistical significance was deemed to be p<0.05.

Results

Small airway wall thickness

The thickness of each small airway wall sub-epithelial area was significantly increased in all pathological groups compared to NC. Specifically, markedly thickened Rbm, LP and adventitia were
observed in COPD-CS (Rbm: median 11.23 \( \mu \)m, range 7.20-15.20 \( \mu \)m, \( p<0.001 \); LP: median 57.95 \( \mu \)m, range 39.72-78.99 \( \mu \)m, \( p<0.001 \); Adventitia: median 107.20 \( \mu \)m, range 56.91-186.20 \( \mu \)m, \( p<0.001 \)) compared to NC (Rbm: median 4.74 \( \mu \)m, range 3.20-11.91 \( \mu \)m; LP: median 21.74 \( \mu \)m, range 10.00-48.37 \( \mu \)m; Adventitia: median 41.23 \( \mu \)m, range 24.01-88.82 \( \mu \)m). In addition, the Rbm thickness was notably high in SAD (median 12.89 \( \mu \)m, range 7.67-17.84 \( \mu \)m, \( p<0.001 \)) compared to NC. NLFS and COPD-ES groups also showed a significant thickness of Rbm (\( p<0.005 \)), LP (NLFS \( p<0.005 \); COPD-ES \( p<0.05 \)) and adventitia (\( p<0.005 \)) (Figure 1).

**EMT markers expression in small airway epithelium**

Representative images of EMT markers' expression in small airways of COPD-CS and NC are shown in Figure 2. Intense positive mesenchymal markers expression (brown colour) was seen in COPD-CS (Figure 2 d., f., h. & j.) compared to NC (Figure 2 c., e. g. & i.), while the epithelial marker was lost in COPD-CS (Figure 2 b.) versus NC (Figure 2 a.). Epithelial marker E-cadherin expression significantly decreased across all pathological groups compared to NC (\( p<0.01 \)) (Figure 3 a). In contrast, a significant increase in mesenchymal marker N-cadherin was observed in SAD (\( p<0.05 \)) and COPD-CS (\( p<0.01 \)) compared to NC (Figure 3 b). Even though the increase in N-cadherin expression was not noteworthy in every pathological group compared to NC, the ratio of N-cadherin to E-cadherin was substantially increased by over twenty-fold in all pathological groups, particularly the COPD-CS group, which showed a twenty-four-fold increase (Figure 4 a). Vimentin and S100A4 epithelial expression were significantly upregulated in all pathological groups compared to NC (\( p<0.05 \), \( p<0.05 \), respectively) (Figure 3 c & d). In addition, the epithelial expression percentage ratio of both Vimentin and S100A4 to E-cadherin has increased by 1.67 to 4.79 folds in all pathological groups (Figure 4 b & c). Similarly, EGFR epithelial expression was markedly increased in all pathological groups compared to NC (\( p<0.05 \)) (Figure 3 e); the expression ratio to E-cadherin also showed a nineteen to forty folds increase across all the pathological groups compared to NC (Figure 4 d).

**Mesenchymal markers expression in Rbm, LP and Adventitia**

Similar to epithelium, Rbm cells were positive for Vimentin and S100A4 across all pathological groups compared to NC (\( p<0.01 \), \( p<0.05 \), respectively) (Figure 5 a & b). Rbm Vimentin and S100A4 expression negatively correlated with E-cadherin expression, respectively (\( r=-1.32 \), \( p<0.005 \); \( r=-0.45 \), \( p<0.05 \)). In addition, compared to NC, Vimentin cell counts (cells/mm\(^2\)) in LP and adventitia showed a significant increase across all pathological groups (\( p<0.01 \), \( p<0.05 \), respectively) (Figure 5 c & e); however, this increase was not observed for S100A4 (Figure 5 d & f). Interestingly, a reverse trend
was observed with Vimentin positive cells in Rbm, LP and adventitia in COPD-ES, which, however, was not observed with S100A4. These indicate the potential for smoking cessation.

**Correlation between EMT markers expression and small airway thickness in COPD**

We have observed a positive correlation between mesenchymal markers and small airway wall thickness in the COPD group. In specific, Vimentin epithelial expression and S100A4 Rbm expression showed a significant positive correlation with Rbm thickness (r’=0.523, p<0.01; r’=0.537, p<0.01, respectively). Vimentin epithelial expression also positively correlated to LP thickness (r’=0.505, p<0.01), and S100A4 epithelial expression positively correlated to adventitial thickness (r’=0.429, p<0.05).

**Correlation between EMT markers and airway wall remodelling factors (α-SMA, fibronectin and collagen-1) in COPD**

Small airway wall remodelling factors in sub-epithelia layers, including α-SMA, collagen-1 and Fibronectin expression data from our previous study [16] were used for correlation analysis. These markers' expressions were positively correlated with EMT markers. Significantly, EGFR epithelial expression was positively correlated with α-SMA expression in RBM (r’=0.685, p<0.02). N-cadherin epithelial and S100A4 RBM expression was positively correlated with collagen-1 expression in LP (r’=0.411, p<0.05; r’=0.375, p<0.05, respectively), and Vimentin epithelial expression was positively correlated to collagen-1 adventitia expression (r’=0.435, p<0.03).

**Correlation between EMT markers expression, smoking history and lung physiology parameters in patients with COPD**

We observed a positive correlation between smoking pack-years and Rbm thickness (r’=0.456, p=0.0249) (Figure 6 a) and similar significant positive correlations of smoking pack-years with Vimentin, S100A4 Rbm expression (r’=0.378, p=0.05; r’=0.475, p=0.0172, respectively) (Figure 6 c & e), and N-cadherin and EGFR epithelial expression (r’=0.452, p<0.01; r’=0.440, p<0.05, respectively) in the COPD groups (Figure 6 b & f). We also observed a close to statistically significant positive correlation between Vimentin epithelial expression and pack-years (Figure 6 d). The EMT markers expression was negatively correlated with lung function FEV1/FVC, particularly S100A4 Rbm and LP expression (r’=-0.410, p<0.05; r’=-0.552, p<0.01, respectively), and EGFR expression was negatively correlated with FEF 25-75% (r’=-0.415, p<0.05). We also found that airway thickness in NLFS and COPD-CS, particularly Rbm and adventitia thickness, negatively correlated with FEV1/FVC (r’=-0.646, p<0.005; r’=-0.649, p<0.005, respectively), and negatively correlated with FEF 25-75% (r’=-0.530, p<0.05; r’=-0.531, p<0.005, respectively).
**Correlation between EMT markers expression and cancer morphology type**

Adenocarcinoma and squamous cell carcinoma phenotypes were more commonly seen in the COPD current smoking patient group (Figure 7 a & b). All mesenchymal markers' airway expression in both cancer phenotypes was significantly higher than NC, N-cadherin and EGFR expression in particular (Figure 7 c). EGFR and S100A4 were relatively higher in squamous cell carcinoma than adenocarcinoma, particularly in COPD-CS (Figure 7 d & e).

**Discussion**

In the current study, we observed small airway remodelling in a more detailed subgroup. Specifically, the thickness of sub-epithelial layers, Rbm, LP and adventitial, were markedly increased in the current smoker with COPD group and without COPD (NLFS) group compared to NC. Although in the ex-smoker with COPD group sub-epithelial layers were significantly thickened compared to normal, it showed a reverse trend compared to the COPD-CS group, but still higher than the NLFS group. This indicated potential for smoking cessation. This work provides basis for investigating effects of longitudinal smoking cessation on EMT, angiogenesis, TGF-β/Smad, Wnt/β-catenin signalling and inflammatory pathways. Such studies will be highly informative in understanding what gets switched off on smoking cessation and what does not, since ex-smokers still get lung cancer and continue to have fibrotic changes.

We further investigate the EMT biomarker expression in each sub-epithelial layer and correlate it with the thickness in these patients. EMT markers in the layers are a sign of cell migration – type III EMT activity. We observed a substantial increase in Vimentin expression in all sub-epithelial layers of the small airway wall in all pathological groups. The most significant increase was observed in the COPD-CS and NLFS groups compared to the NC group. Additionally, we found a robust positive correlation between Vimentin expression and Rbm and LP thickness.

Our findings indicated a significant increase in S100A4 or fibroblast specific protein-1 levels within the small airway epithelium and Rbm of both COPD groups and NLFS compared to NC. S100A4 expression positively correlated with Rbm and adventitial thickness. The correlation between Vimentin, S100A4 and airway thickness is further supported by the positive correlation with airway wall remodelling factors, α-SMA, fibronectin and collagen-1 expression in sub-epithelial layers. Indeed, the airway epithelium of COPD patients has exhibited heightened cellular expression of mesenchymal markers, such as S100A4 and Vimentin [17]. We have previously shown that EMT increased myofibroblasts, leading to the formation and progression of fibrosis in COPD patients [16, 22]. EMT-mediated fibrosis is also one of the causes of lung cancer [23]. Therefore, the prevalence of
lung cancer among COPD patients is primarily attributed to the presence of airway fibrosis and remodelling [24, 25]. Additionally, the elevated levels of S100A4 and Vimentin were inversely correlated with lung function, indicating a potential physiological impact [26].

Furthermore, the other main characteristic of EMT is the decrease in E-cadherin levels. E-cadherin is crucial for preserving the connection between epithelial cells and arranging the cytoskeleton [27]. On the other hand, N-cadherin is an intercellular junctional mesenchymal marker. The presence of N-cadherin suggests the occurrence of EMT, and its expression has been linked to the progression of different types of carcinomas [28-30]. We observed a marketable decrease in E-cadherin across all pathological groups, along with an increase in N-cadherin, with a particularly high increase in the COPD-CS group compared to the NC group. The ratio of N-cadherin to E-cadherin in the COPD-CS group was approximately twenty-four-fold higher, indicating that small airway epithelium lost the epithelial characteristics and transformed to mesenchymal phenotype in all pathological groups. The negative correlations of the epithelial Vimentin and S100A4 expression to E-cadherin expression further indicated the mesenchymal transition progressing (data is not shown here). In addition, we observed that N-cadherin was significantly elevated, whereas E-cadherin was significantly downregulated in both carcinoma phenotypes compared to NC, and N-cadherin was higher than other mesenchymal makers – Vimentin and S100A4. In cancer-related EMT, it involves an increased expression of N-cadherin and a decreased expression of E-cadherin. This shift in cadherin expression is linked to augmented migratory and invasive characteristics, leading to lower patient survival rates [28, 31].

Cigarette smoking is a significant risk factor for developing lung cancer and COPD. Our results showed that Vimentin and S100A4 were markedly increased in the NLFS group compared to NC and even higher in smokers with COPD. The expression of N-cadherin, Vimentin and S100A4 were also positively correlated with smoking history, which further indicates that EMT progression is escalated due to smoking and COPD. More EMT activity was seen in squamous cell carcinoma compared to adenocarcinoma, especially in current smokers with COPD. On the other hand, the EMT activity was relatively lower in COPD-ES compared to COPD-CS. This indicates that smoking cessation could potentially reduce the EMT progress, therefore, prevent cancer development.

EGFR is implicated in the pathogenesis of NSCLC [32, 33]. Our results demonstrated significantly elevated EGFR levels in smokers with and without COPD, particularly high in COPD-CS, and associated with a strong positive correlation with smoking history. Moreover, the EGFR expression was higher in both NSCLC morphological types and significantly higher in COPD-CS with squamous
cell carcinoma. The heightened expression of EGFR in NSCLC is related to lower survival rates [32], as well as frequent lymph node metastasis and inadequate response to chemotherapy.

Enhanced understanding of EMT mechanisms linked to diseases showed an opportunity for precise therapy aimed at inhibiting COPD progression and preventing cancer metastasis. Inhaled corticosteroids (ICS) have become a standard treatment in more severe COPD and have an anti-EMT effect in COPD airways [34, 35], further reducing the risk of lung cancer in COPD patients [36], which suggests administering ICS or other medications with comparable effects at an early stage of COPD [37]. This can not only help to reduce airway inflammation but also prevent EMT activation, resulting in fibrosis and potential malignant consequences. In addition to EMT, we believe endothelial to mesenchymal transition (EndMT) can also contribute to the formation of pro-cancer stroma and fibrosis (Figure 8). During EndMT, like epithelial cells, endothelial cells can also gain a mesenchymal phenotype leading to fibrotic or malignant changes [25]. Further work in this direction would be of great value.

There are limitations to this study. Firstly, the sample size is relatively small, which limits the correlation evaluation. For example, we can see the negative correlation trend between EMT markers expression and lung function; however, a larger sample size can improve the outcomes and better evaluate the EMT activity-mediated lung physiology changes. Secondly, the clinical and pathological stage of lung cancer is unavailable. However, our findings have indicated that escalated EMT-mediated fibrosis and small airway remodelling in early COPD contributes to NSCLC development.

In conclusion, this study investigated EMT activities in lung cancer patients with COPD. We found that EMT is an active process in the small airway of smokers with COPD diagnosed with lung cancer, contributing to small airway remodelling and cancer development seen in these patients. This is the first study to show such changes in broadly phenotyped individuals. EMT markers and EGFR can be used as the therapeutic target in lung cancer patients with COPD, and smoking cessation can assist in reducing the EMT progress [38-40].

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**Conflict of Interest:** Dr. Sohal has served on the Small Airway Advisory Board for Chiesi Australia and have received the honorarium, outside the submitted work. Dr Sohal reports travel support from Chiesi, GSK and AstraZeneca, outside the submitted work. All the other authors do not have any conflict of interest to declare.
## Table 1. Patient demographics

### Patient Demographics (Adenocarcinoma n=22; Squamous cell carcinoma n=13)

<table>
<thead>
<tr>
<th>Groups</th>
<th>NC</th>
<th>NLFS</th>
<th>SAD</th>
<th>COPD-CS</th>
<th>COPD-ES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>11</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>GOLD I/II</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>3/6</td>
<td>6/4</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>6/5</td>
<td>4/3</td>
<td>7/2</td>
<td>5/4</td>
<td>4/6</td>
</tr>
<tr>
<td>Age (median, range)</td>
<td>47.35-87</td>
<td>72.52-79</td>
<td>59.42-84</td>
<td>63.59-78</td>
<td>68.56-85</td>
</tr>
<tr>
<td>Smoking pack-years (mean ± SD)</td>
<td>NA</td>
<td>20.69 ± 21.44</td>
<td>35.67 ± 20.54</td>
<td>32.25 ± 15.81</td>
<td>28.8 ± 17.78</td>
</tr>
<tr>
<td>FEV1 % Pred Post BD (mean ± SD)</td>
<td>NA</td>
<td>107.5 ± 22.98</td>
<td>86.4 ± 21.8</td>
<td>82.14 ± 12.52</td>
<td>83.68 ± 11.29</td>
</tr>
<tr>
<td>FEV1/FVC% Post BD (mean ± SD)</td>
<td>NA</td>
<td>79.7 ± 6.45</td>
<td>73.84 ± 3.21</td>
<td>66.39 ± 3.55</td>
<td>63.39 ± 4.83</td>
</tr>
<tr>
<td>FEF 25-75% (L/sec) Post BD (mean ± SD)</td>
<td>NA</td>
<td>86.64 ± 16.4</td>
<td>46.6 ± 12.78</td>
<td>35.63 ± 6.26</td>
<td>39.84 ± 11.52</td>
</tr>
</tbody>
</table>

Abbreviations: NC – normal control; NLFS – normal lung function smoker; SAD – small airway disease; COPD-CS – COPD current smoker; COPD-ES – COPD ex-smoker; GOLD – Global Initiative for Chronic Obstructive Lung Disease; FEV1% Pred Post BD – force expiration % predict post-bronchodilator; FEV1/FVC % Post BD – forced expiration / forced vital capacity% post-bronchodilator; FEF 25-75% (L/sec) Post BD – forced expiratory flow at 25-75% post-bronchodilator.
References


**Figure 1:** Small airway sub-epithelial layer thickness across all pathological groups compared to NC. a). Rbm thickness; b). LP thickness; c). Adventitia thickness. Abbreviation: Rbm – reticular basement membrane; LP – lamina propria. NC – normal control; NLFS – normal lung function smoker; SAD – small airway diseases; COPD-CS – chronic obstructive pulmonary disease current smoker; COPD-ES – chronic obstructive pulmonary disease ex-smoker. * p<0.05, ** p<0.01, *** p<0.005, **** p<0.001.

**Figure 2:** Representative images of EMT markers’ expression in COPD-CS and NC small airway. a) & b). E-cadherin expression; c) & d). N-cadherin expression; e) & f). Vimentin expression; g) & h). S100A4 expression; i) & j). EGFR expression. Abbreviations: NC – normal controls; COPD-CS – COPD current smokers; EGFR – epidermal growth factor receptor. Bright-field 40x.

**Figure 3:** EMT markers’ expression in small airway epithelium. a). E-cadherin expression percentage; b). N-cadherin expression percentage; c). Vimentin expression percentage; d). S100A4 expression percentage; e). EGFR expression percentage. Abbreviations: SA – small airway; NC – normal control; NLFS – normal lung function smoker; SAD – small airway disease; COPD-CS – COPD current smoker; COPD-ES – COPD ex-smoker; EGFR – epidermal growth factor receptor; EP – epithelium. *p<0.05; **p<0.01; ***p<0.005; ****p<0.001.

**Figure 4:** EMT markers’ expression ratio in small airway epithelium. a). the expression ratio between N-cadherin and E-cadherin; b). the expression ratio between Vimentin and E-cadherin; c). the expression ratio between S100A4 and E-cadherin; d). the expression ratio between EGFR and E-cadherin. Abbreviations: SA – small airway; NC – normal control; NLFS – normal lung function smoker; SAD – small airway disease; COPD-CS – COPD current smoker; COPD-ES – COPD ex-smoker; EGFR – epidermal growth factor receptor.

**Figure 5:** EMT markers’ expression in small airway sub-epithelial layers. a). Vimentin-positive cells per mm of Rbm length in Rbm; b). S100A4-positive cells per mm of Rbm length in Rbm; c). Vimentin-positive cells per mm² of LP area in LP; d). S100A4-positive cells per mm² of LP area in LP; e). Vimentin-positive cells per mm² of adventitia area in adventitia; f). S100A4-positive cells per mm² of adventitia area in adventitia. Abbreviations: SA – small airway; NC – normal control; NLFS – normal lung function smoker; SAD – small airway disease; COPD-CS – COPD current smoker; COPD-ES – COPD ex-smoker; Rbm – reticular basement membrane; LP – lamina propria. *p<0.05; **p<0.01; ***p<0.005; ****p<0.001.

**Figure 6:** Correlation between EMT markers express and smoking history. a). Rbm thickness versus smoking packs; b). N-cadherin expression percentage versus smoking packs; c). Vimentin Rbm
expression versus smoking packs; d) Vimentin epithelial expression versus smoking packs; e) S100A4 Rbm expression versus smoking packs; f) EGFR epithelial expression versus smoking packs. Abbreviations: EGFR – epidermal growth factor receptor; pack/yr – packs per year; Rbm – reticular basement membrane.

**Figure 7:** EMT markers expression and cancer morphological types. a) the proportion of each subgroup with adenocarcinoma phenotype; b) the proportion of each subgroup with squamous cell carcinoma phenotype. a) and b) indicate COPD-CS is the major proportion in both cancer type. c) EMT markers' expression percentage in adenocarcinoma and squamous cell carcinoma compared to NC; d) EGFR expression of each subgroup in adenocarcinoma versus squamous cell carcinoma; e) S100A4 expression of each subgroup in adenocarcinoma versus squamous cell carcinoma. Red circles in d) and e) indicate EGFR and S100A4 were relatively higher in squamous cell carcinoma in COPD-CS than adenocarcinoma, respectively. Abbreviations: NC – normal control; NLFS – normal lung function smoker; SAD – small airway disease; COPD-CS – COPD current smoker; COPD-ES – COPD ex-smoker; EGFR – epidermal growth factor receptor; SCC – squamous cell carcinoma. *p<0.05; ** p<0.01.

**Figure 8:** Cigarette smoking activates the TGF-β/SMAD, Wnt/β-catenin and EGFR signalling pathways. Both pathways initiate the epithelial-mesenchymal transition (EMT) and endothelial-mesenchymal transition (EndMT) process in the airways and vasculature. During EMT, there is a reduction in the expression of epithelial junctional protein – E-cadherin, resulting in a loss of cell-cell adhesion among epithelial cells. Simultaneously, these cells acquire mesenchymal phenotype and proteins, including N-cadherin, Vimentin and S100A4, leading to the formation of fibrotic tissue and pro-cancer stroma. Similarly, in EndMT, endothelial cells lose endothelial junctional proteins, such as VE-cadherin and CD31, transforming into mesenchymal cells such as fibroblasts, resulting in vascular remodelling, fibrosis, and cancer. EMT and EndMT in COPD can both create the pro-cancer stroma that promotes lung cancer development, including both squamous cell and adenocarcinoma.
Figure 1
Figure 2
Figure 3
Figure 4

(a) E-Cadherin vs N-Cadherin expression ratio in SA

(b) E-Cadherin vs Vimentin epithelium expression ratio in SA

(c) E-Cadherin vs S100A4 epithelium expression ratio in SA

(d) E-Cadherin vs EGFR expression ratio in SA
Figure 5

(a) Vimentin expression in SA Rbm

(b) S100A4 expression in SA Rbm

(c) Vimentin expression in SA LP

(d) S100A4 expression in SA LP

(e) Vimentin expression in SA Adventitia

(f) S100A4 expression in SA Adventitia
Figure 6
Figure 7

**a.** Adenocarcinoma

- SAD: 23.35%
- COPD-CS: 43.28%
- COPD-ES: 19.69%
- NLFS: 13.69%

**b.** Squamous cell carcinoma

- SAD: 30.65%
- COPD-CS: 42.08%
- COPD-ES: 24.53%
- NLFS: 2.74%

**c.** NC vs Adenocarcinoma vs SCC

- E-Cadherin
- N-Cadherin
- EGFR
- Vimentin
- S100A4

**d.** EGFR expression in Adenocarcinoma vs Squamous cell carcinoma

**e.** S100A4 expression in Adenocarcinoma vs Squamous cell carcinoma

Figure 7
Figure 8