Research letter

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Large airway wall vascularity in patients with Asthma COPD overlap: a bronchoscopy study

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To The Editor

Asthma COPD overlap (ACO) is a clinical term that describes patients with persistent airflow limitation, and clinical/physiologic characteristics consistent with both asthma and chronic obstructive pulmonary disease (COPD) [1]. The hallmark pathological features of asthma and COPD are airway inflammation and remodelling. These have been considered as separate processes or perhaps as sequential, with early inflammation leading later to remodelling; however, a little is known about these parameters in ACO. We have recently reported increase in inflammatory cells such as macrophages and CD8+ T cells in the airway mucosa of patients with ACO [2]. Notably, our previous findings of airway remodelling in ACO airway suggested a thicker reticular basement membrane (RBM) and higher RBM cellularity [3]. Another crucial element of tissue remodelling is angiogenesis. In asthma, a majority of studies reported an increase in tissue vascularity, while in COPD there are conflicting reports on airway tissue vascularity contributing to disease [1, 4-8]. In this cross-sectional exploratory study, we hypothesise that tissue vascularity in the airway of ACO could be different from that of asthma, COPD, and healthy controls. To evaluate this, we analysed large airway endobronchial biopsies from patients with ACO and compared them against healthy controls (HC), patients with asthma, COPD ex-smokers (ES) and current smokers (CS), and normal lung function smokers (NLFS).

We immunohistochemically stained 3 µm thick bronchial biopsy sections following optimisation. The tissues were obtained from biobank and were collected from physician diagnosed patients (diagnostic criteria previously published by our team [2, 3]) with ACO, asthma, COPD, and HC, and NLFS. The patients with ACO in our study were ex-smokers, our biobank did not have any tissue available from current smokers with ACO. A representative tissue micrograph and counting strategy is provided in
Figure 1A and the patient demography and baseline information is provided in Figure 1B. Primary rabbit polyclonal anti-Collagen IV (1:350, ab6586, Abcam, Victoria, Australia) was used. Following staining, computer-assisted image analysis was performed as previously described [2, 3]. Stained tissues with visible epithelium, RBM, and LP were selected for image analysis. The images of the entire tissue area, including epithelium, RBM and LP, were captured at 40X brightfield, avoiding the overlapping area between images. From five randomly selected images, collagen IV-positive vessels were counted in the epithelium, RBM, and in the LP i.e., 120 µm deep inside the tissue (Figure 1A). The observer was blinded to patient and diagnosis. The epithelium and RBM-associated vessels were presented as per mm of RBM length, and the vessels in the LP were presented as per mm² of the LP area [9, 10]. Vessel count data distribution was evaluated using D’Agostino & Pearson test, and intra- and inter-group variances were analyzed Kruskal–Wallis (nonparametric) with multiple comparisons using uncorrected Dunn's test.

Overall, the number of epithelial vessels in ACO (Figure 1C) were similar to the HC and asthma and tended to be higher than the COPD-ES and NLFS. The number of epithelial vessels in COPD-CS tended to be increased and in COPD-ES the vessel numbers tended to be decreased when compared with HC; however, the difference was not statistically significant (P = 0.9891 and 0.4412, respectively).

The number of RBM vessels in patients with ACO (Figure 1D) were significantly higher (P <0.05) than the HC. Furthermore, the number of RBM vessels in ACO appeared to be higher than asthma (P = 0.2316), COPD-ES (P = 0.7881), and NLFS (P = 0.9201); however, the difference was statistically not significant. A similar increase in the number of vessels was noted between the patients with ACO and COPD-CS. We have also noted a statistically significant increase in the number of RBM vessels (P
<0.05) in COPD-CS compared to HC. Although the asthma group appeared to have higher RBM vessels than the HC, the difference was not statistically significant ($P = 0.3180$). Among asthma and COPD groups, the RBM vessels were greatest in COPD-CS which was statistically significant ($P <0.05$) than asthma. Furthermore, RBM vessels in COPD-CS group also tended to be higher than the COPD-ES ($P = 0.4726$) but was not statistically significant.

In contrast to the RBM, we observed the lowest number of vessels in the LP area of patients with ACO which was statistically significant ($P <0.01$) than the HC (Figure 1E). In addition, the number of LP vessels in ACO tended to be lower (than the COPD-ES ($P = 0.1128$) and NLFS ($P = 0.1633$); however, the difference was not statistically significant. Furthermore, we noted similar RBM vessel numbers in ACO, asthma and COPD-CS groups. The number of LP vessels in asthma and COPD-CS were also lower ($P <0.05$ and <0.01, respectively) than the HC. Furthermore, the number of LP vessels in COPD-ES group appeared to be lower than the HC ($P = 0.1351$) but the difference was not statistically significant.

We checked the effect of ICS treatment in by dichotomizing the vessel numbers in ACO patients with and without ICS treatment, and the results suggested a trend of lower numbers of vessels in the RBM ($P = 0.5049$) and LP ($P = 0.2132$) of ICS treated patients as compared with not on ICS (median difference: 0.71/mm and 54.51/mm$^2$, respectively) although was not statistically significant, suggesting ICS treatment effect on number of vessels. However, in COPD we reported previously that inhaled fluticasone propionate normalises LP vascularity and reduces RBM cellularity [10, 11]. Furthermore, the correlation analysis between the RBM vessels and RBM thickness was not significant in patients with ACO (spearman $r$ -0.0818, $P = 0.4090$) or FEV1/FVC (spearman $r$ 0.1888, $P = 0.2788$).
Despite limited sample size, this cross-sectional study provides valuable insights on tissue vascularity in the mucosa of patients with ACO comparing with contributing diseases, smokers, and HC. We believe that these are the first observational findings of tissue vascularity in patients with ACO. Our findings suggest a prominent and contrasting change in RBM and LP vascularity in ACO patients compared to HC with a similar observation for contributing diseases of asthma and COPD. Soltani et al reported hyper vascular RBM with higher vessel permeability and hypo vascular LP in smoker COPD patients [4, 9]. Kuwano et al reported a similar vascular area and vessel dimension in submucosa between COPD and HC [12]. Increased tissue vascularity were also reported in the submucosal area of patients with asthma [6, 12] and possibly due to neovascularisation or angiogenesis in response to local inflammation and growth factors elaboration [13] causing more blood flow possibly to meet increased metabolic need. Although hypothetical at this stage but high RBM vascularity in ACO could be due to higher cellular activities with respect to epithelial mesenchymal changes (EMT) that we previously reported in COPD [1, 3, 14]. ACO being the overlap of asthma and COPD disease, theoretically, it will not be wrong to state that some of the established pathology of tissue vascularity of either disease may also be active in ACO, as evident from our vascularity data on ACO. With respect to effect of ICS on tissue vascularity in patients with ACO, it is well established that ICS has an effect on the reduction of tissue vascularity in asthma [15], our ICS data shows a similar trend in ACO, with 6/12 subjects on ICS.

Our study has the limitation of not comparing the vascular area among the groups. In addition, one might think the age could be confounder; however, we have not noticed a consistent pattern of correlation between age and the vessel count among the groups. For some of our findings we only noticed trends without statistically significant
differences due to a small sample size. Future studies are needed with larger cohorts. In addition, our findings from this study were limited to patients with ACO who were ex-smokers. However, one can expect that current smoking will only lead to a more destructive pathology.

Taken together, our findings of high RBM vascularity in the large airway wall of ACO and decrease in LP vascularity are novel and indicate COPD like pathology [9]. In COPD increased vascularity is attributed to EMT changes leading to formation of pro-cancer stroma and airway fibrosis. EMT could also be an active process in patients with ACO but warrants further investigations. We believe our findings will enhance clinical understanding on ACO helping physicians with informed decision making.

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**Conflict of interest:** S.S. Sohal reports honorarium for lectures from Chiesi, travel support from Chiesi, AstraZeneca and GSK, and research grants from Boehringer Ingelheim and Lung Therapeutics, outside the submitted work; and has served on the small airway advisory board for Chiesi Australia for which an honorarium has been received.
References

Figure 1: Tissue micrograph (A) showing Collagen IV positive vessels in the epithelium (yellow arrow), RBM (green arrow), and LP area (black arrow). Patient demographics are presented in panel B. Seventy large airway endobronchial biopsy samples were collected from the participants and tissue were obtained from the Tasmanian Respiratory Tissue Bank and Newcastle Biobank (Tasmanian Health and Medical Human Research Ethics Committee, ethics ID: H0013051; the Hunter New England Human Research Ethics Committee reference no: 05/08/10/3.09). In panel C, D, and E, box plots are presented showing epithelial vessels per mm of reticular basement membrane (RBM), RBM vessels per mm of RBM, and lamina propria vessels/mm² area, respectively, in HC, ACO, asthma, chronic COPD-ES and COPD-CS, and NLFS. Triangles represents the vessel counts in patients treated with ICS. The horizontal line inside each box represents the median; the top and bottom of each box represent the upper and lower quartiles, respectively; and the whiskers represent extreme values. Multiple comparison $P$ value representation * $<0.05$ and ** $<0.01$. 
Figure 1