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RESEARCH ARTICLE

Differential roles for lysyl oxidase (like), family members in chronic obstructive pulmonary disease; from gene and protein expression to function

Nataliya Migulina[1] | **Gavin Tjin**[2,3,4] | **Alen Faiz**[1,5] | **Theo Borghuis**[1] | **Fenghua Zhao**[6,7] | **Hans J. Kaper**[6,7] | **Marit Metzlar**[8] | **Eline van Dijk**[8] | **Prashant K. Sharma**[6,7] | **Wim Timens**[1] | **Reinoud Gosens**[8] | **Corry-Anke Brandsma**[1] | **Janette K. Burgess**[1,9]

[1]Department of Pathology and Medical Biology, Groningen Research Institute for Asthma and COPD, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

[2]Respiratory Cellular and Molecular Biology, Woolcock Institute of Medical Research, Sydney, New South Wales, Australia

[3]Discipline of Pharmacology, School of Medical Sciences, The University of Sydney, Sydney, New South Wales, Australia

[4]Central Clinical School, The University of Sydney, Sydney, New South Wales, Australia

[5]Faculty of Science, Respiratory Bioinformatics and Molecular Biology, University of Technology Sydney, Ultimo, New South Wales, Australia

[6]W.J. Kolff Institute for Biomedical Engineering and Materials Science-FB41, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

[7]Department of Biomedical Engineering-FB40, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

[8]Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands

[9]Department of Pathology and Medical Biology, KOLFF Institute, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Correspondence
Nataliya Migulina, Department of Pathology and Medical Biology, University Medical Center Groningen, Hanzeplein 1, IPC EA11, Rm T2.212, Groningen 9713 GZ, The Netherlands.
Email: n.migulina@umcg.nl

Present address
Gavin Tjin, St Vincent's Institute Medical Research, Fitzroy, Victoria, Australia

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Abstract

Chronic obstructive pulmonary disease (COPD) is characterized by long-term airflow obstruction with cigarette smoke as a key risk factor. Extracellular matrix (ECM) alterations in COPD may lead to small airway wall fibrosis. Altered collagen cross-linking, potentially mediated by the lysyl oxidase (LO) family of enzymes (LOX, LOXL1-4), orchestrates disturbed ECM homeostasis. In this study, we investigated the effects of smoking status and presence and severity of COPD on LOs gene and protein expression in the airways and the impact of LOs inhibition on airway contraction in an ex vivo mouse model. We used gene expression data from bronchial brushings, airway smooth muscle (ASM) cells in vitro and immunohistochemistry in lung tissue to assess smoke- and COPD-associated differences in LOs gene and protein expression in the small airways. We found

Abbreviations: ASM, airway smooth muscle; BAPN, β-aminopropionitrile; COPD, chronic obstructive pulmonary disease; Cq, chloroquine; CSE, cigarette smoke extract; DAB, 3,3′-diaminobenzidine; ECM, extracellular matrix; IHC, immunohistochemistry; IPF, idiopathic pulmonary fibrosis; LO, lysyl oxidase family of enzymes; LOX, lysyl oxidase; LOXL, lysyl oxidase-like protein; MCh, methacholine; SHG, second harmonic generation; μLLCT, micro-low-load compression tester.

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higher *LOX* expression in current- compared to ex-smokers and higher *LOXL1* expression in COPD compared to non-COPD patients. *LOX* and *LOXL2* expression were upregulated in COPD ASM cells treated with cigarette smoke extract. LOXL1 and LOXL2 protein levels were higher in small airways from current- compared to non-smokers. In COPD patients, higher LOXL1 and lower LOX protein levels were observed, but no differences for LOXL2, LOXL3, and LOXL4 protein were detected in small airways. Inhibiting LOs activity increased airway contraction in murine lung slices. COPD-associated changes in LOs, in particular LOX and LOXL1, may be related to smoking and contribute to impaired airway function, providing potential novel targets for preventing or treating small airways changes in COPD.

KEYWORDS

collagen, COPD, ECM, lysyl oxidase, smoking

1 | INTRODUCTION

According to the World Health Organization, in 2019, chronic obstructive pulmonary disease (COPD) caused 6% of all deaths globally (more than 3 million annually) and became the third leading cause of death.[1] Chronic exposure to toxic substances including cigarette smoke is an important risk factor for the development of COPD. The structural changes in the airways in patients with COPD include epithelial cell metaplasia, increase in goblet cell or mucus producing cell numbers, changes in airway smooth muscle (ASM) and altered composition of the extracellular matrix (ECM) in the airway wall.[2–4]

The most abundant ECM proteins in the lung are fibrillar collagens (Types I, II, III, V, and XI),[5] which provide structural support and have crucial bioactive roles including regulation of cellular attachment, migration, differentiation and cytokine production.[6–8] Alterations of the collagen structure have recently been reported in alveoli[2] and large airways[4] of subjects with COPD. Although it is known that collagen deposition is increased in the ECM of the small airway walls in COPD, little is known about the mechanisms changing the structure of this collagen and the possible effects on physiology.[9]

The stabilization of collagen fibers, and thus their mechanical properties, are partially dependent on the level of intermolecular cross-linking within these fibers. While the intermolecular fibrillar organization takes place spontaneously, proteoglycans like decorin and enzymes such as lysyl oxidases[10] are crucial for covalent cross-linking to occur and subsequently affect the mechanical properties of the collagen matrix. The LO family of enzymes are secreted, copper-dependent amine oxidases consisting of five known paralogues (LOX and lysyl oxidase like proteins (LOXL)-1-4). The expression of LOs is tightly regulated during normal development, with aberrant expression and activity being associated with various diseases including cancer and idiopathic pulmonary fibrosis (IPF).[11] The deficiency of LO activity, as seen in Menkes disease, results in the development of severe diffuse emphysema, leading to respiratory failure and early death.[10] The LO family of enzymes is often considered as a whole; however, each of the family members has individual, occasionally opposite, functions. Interestingly, differential expression patterns for LO family members have been reported in lung diseases. For example, decreased LOX levels, but increased LOXL1 and LOXL2 production have been associated with IPF,[12] whereas the role of LOXL3 and LOXL4 in lung tissue is not well studied.[13] Whether the expression of LOs is also changed in COPD and is involved in the structural changes in the small airways of patients with COPD is unknown. Furthermore, limited information is available on the effects of cigarette smoking, a key risk factor of COPD, on expression of LOs in human lung tissue. In this study, we investigated the effects of smoking status and the presence and severity of COPD on the gene and protein expression of LOs in the (small) airways and its functional relationship with contraction of the small airways in an ex vivo mouse model.

2 | MATERIALS AND METHODS

2.1 | Human tissues used for IHC

Human lung tissue was obtained from subjects undergoing surgery for resection of lung tumors or lung transplantation for severe COPD at UMCG. Lung function was assessed prior to surgery, and subjects were classified as non-COPD control, Global Initiative for Obstructive

Lung Disease (GOLD) stage II (moderate COPD) and stage IV (severe COPD). Non-COPD control was defined as forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) > 70% and $FEV_1 \geq 80\%$, GOLD stage II was defined as FEV1/FVC < 70% and 50% < FEV_1 < 80% of the predicted value, and GOLD stage IV as FEV1/FVC < 70%; 30% < FEV1 < 50% predicted. To avoid possible effects of the tumor on the samples, the lung tissues were obtained as distant from the tumor as possible. In all groups peripheral lung tissues were used following a protocol that was consistent with the Research Code of the University Medical Center Groningen, (https://umcgresearch.org/en/web/research/w/research-code-umcg) and the Dutch national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies"; http://www.federa.org).[14,15]

2.2 | Gene expression analysis of bronchial brushings

2.2.1 | Data acquisition, probeset and normalization

Publicly available data from affymetrix_Hu_gene_st1.0 arrays from current and ex-smoking COPD patients ($n = 87$) and controls ($n = 151$) (GSE37147)[16] were used to examine differential expression of LOs in relation to smoking behavior and presence of COPD. Normalization was performed with R statistical software V3.0.2. using the Robust Multichip Analysis (RMA) sketch algorithm workflow, and analysis was conducted with the R package Limma (v3.13). The difference between COPD and control was assessed using a linear model adjusting for smoking status, age, and sex as possible confounding variables. Second, we investigated the change in gene expression between current and ex-smokers in non-COPD patients ($n = 238$). This analysis was performed using a linear model with smoking defined as a categorical variable with two levels (1 = current smoking, 2 = ex-smoking) adjusting for disease status, age and gender. Last, we investigated the association between bronchial brushing gene expression and FEV1% predicted ($n = 238$). To this end, a linear regression analysis was performed adjusting for smoking status as a possible confounding variable.

2.3 | Histology and immunohistochemistry

Immunohistochemistry (IHC) staining for LOX and LOXL1-4 was performed on 3 μm thick serial sections of formalin-fixed paraffin-embedded lung tissue using a standard protocol and incubated with the different polyclonal rabbit anti-human LOs antibodies. Antigen retrieval was performed in Tris/EDTA buffer for LOX and LOXL1 staining, and citrate-based buffer for LOXL2-4 staining by incubating the sections for 15 min in these buffers in a microwave. The sections were incubated overnight at 4°C with primary antibodies: rabbit anti-LOX (Abcam, 1.0 mg/ml), rabbit anti-LOXL1 (Novus Biologicals, 0.3 mg/ml), rabbit anti-LOXL2 (Novus Biologicals, 0.68 mg/ml), rabbit anti-LOXL3 (Sigma-Aldrich, 0.1 mg/ml), rabbit anti-LOXL4 (Sigma-Aldrich, 0.1 mg/ml). After washing, the sections were incubated with a secondary goat anti-rabbit and tertiary horseradish peroxidase conjugated rabbit anti-goat antibody (DAKO) each for half an hour at room temperature. Staining was visualized using 3,3′-diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin (Sigma Aldrich).

2.4 | Image analyses

Whole tissue sections were scanned using a NanoZoomer XR digital slide scanner (Hamamatsu Photonics). Fiji ImageJ[17] was used to quantify the density and distribution of staining. The different input settings were optimized per antibody. Color deconvolution[18] vectors in Fiji ImageJ were optimized to ensure accurate separation of hematoxylin and DAB positive pixels. Macros were used to process the images. Overlaid threshold images of hematoxylin and DAB were used to measure the total number of positive pixels as a measure of total stained tissue surface area. The image analyses calculated the number of pixels above the threshold within the image (area of positive pixels) and the average intensity of the pixels above the threshold (average staining intensity). All airways that were present in the lung tissue sections were selected for the analyses (Figure 1A,B). Within each airway, three regions of the interest were selected: the small airways as a whole (Figure 1C), the ASM (Figure 1D) and the epithelial layer (Figure 1E) within the airways. ImageScope (Leica Biosystems, v12.3.0.5056) was used to measure the area of each analyzed region, which was used for the normalization of staining to facilitate comparison of regions with different sizes. The length of the basement membrane (P_{BM}) seen in the airway cross-section and the area of ASM bundle was measured along the length of the outer edge of the ASM bundles. Data of LO enzymes expression in Figures 5 and 6 were represented as:

$$\frac{(\text{DAB area} \times \text{DAB density})}{(\text{length of the } P_{BM} \text{ or area ASM bundle})}$$

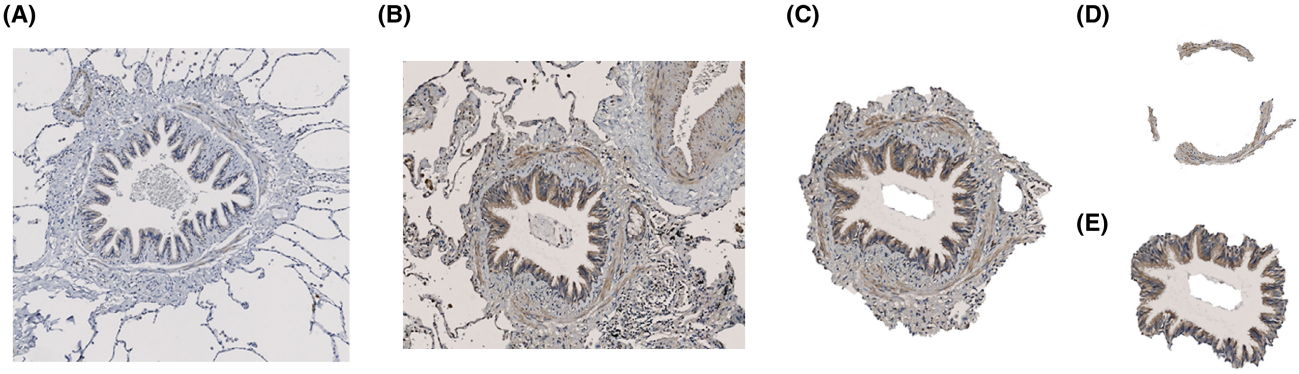

FIGURE 1 Image analysis of specific structures in the human airways. Representative images of immunohistochemically stained human airway tissues from non-COPD non-smoker (A) and COPD smokers (B) (brown = protein of interest; pink = cytoplasm, blue = nuclei). Regions analyzed were total airway (C), airway smooth muscle (D), airway epithelial layer measured from basement membrane (E).

TABLE 1 Patient demographics table

	Control	COPD
n	5	5
Sex	1(f)/4(m)	1(f)/4(m)
Age (±SD) (years)	64.4 (3.7)	63.2 (8.48)
Smoking status	4(ex)/1(never)	N/A

Note: Data presented as mean ± standard deviation (SD).

Abbreviations: COPD, chronic obstructive lung disease; ex, ex-smokers; f, female; m, male; never, never smokers.

2.5 | Human ASM cells used for cigarette smoke exposure

Human ASM cells were isolated from lung tissue derived from subjects undergoing resection surgery for either lung transplantation or carcinoma using a method modified from that described previously.[19,20] Subjects were classified as follows according to severity of airflow limitation[21] (1) non-COPD; $FEV_1/FVC \geq 70\%$ and $FEV_1 \geq 80\%$; (2) COPD; $FEV_1/FVC < 70\%$. Patients with asthma, infectious diseases or interstitial lung diseases were not included. All study subjects or their next of kin provided written informed consent. Approval of all the experiments using human lung tissues was provided by the Ethics Review Committee of the South West Sydney Area Health Service, St Vincent's Hospital Sydney, and the University of Sydney Human Research Ethics Committee.

ASM cells were obtained from 10 subjects; 5 non-COPD control subjects (4 ex-smokers and 1 current smoker) and 5 subjects with COPD. No significant differences were found between age and gender for all groups. Subject demographics are in Table 1.

2.5.1 | Cell culture and sample preparation

Human ASM bundles were micro-dissected from bronchi of approximately sixth-order or greater, and were initially cultured in growth medium Dulbecco's modified eagle's medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (FBS) (DKSH), 1% Penicillin-Streptomycin (P/S) (Invitrogen) and 25 mM (4-(2-hydroxyethyl)-1-pipe razineethanesulfonic acid (HEPES; Invitrogen)) to allow outgrowth of the ASM cells. All the cells tested negative for the presence of mycoplasma before they were set up for experiments and were used between passages 2 and 7.

2.6 | Cell culturing and cigarette smoke extract (CSE) stimulation

ASM cells were cultured in DMEM with 25 mM HEPES (Thermo Fisher), 10% fetal calf serum (FCS), 2% P/S, 1% L glutamine, 250 μg/ml amphotericin and incubated at 5% CO_2, 37°C. After cells reached confluence, they were trypsinized and seeded in six-well culture plates (Corning®) at a density of 1×10^4 cells/cm^2 and incubated at 37°C 5% CO_2 for 24 h. After 24 h, the media was changed to DMEM with 25 mM HEPES (Thermo Fisher), 0.1% BSA, 1% P/S and 1% L-glutamine, for 48 h.

100% solution of CSE was prepared by bubbling smoke from two de-filtered, 1R3 cigarettes (University of Kentucky) at a constant rate (62 rpm pump setting) through 25 ml DMEM containing 25 mM HEPES (Thermo Fisher), 1% P/S, 1% L-glutamine. Then 100% CSE solution was diluted in DMEM and applied to the cells with concentration of 0%, 1%, 5%, and 10%, within 30 min after preparation. ASM cells were incubated with CSE for 6 and 24 h. After incubation, ASM cells were

lysed with 500 μl of Trizol Tri reagent TR-118 (MRC) and stored at −80°C.

2.7 | Gene expression analysis of ASM

RNA was purified according to standard procedure by using Trizol.[22] cDNA was synthesized from RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher) according to the manufacturer's protocol. Solution containing 500 ng RNA sample and Master Mix in a total volume of 20 μl per sample was placed in a PCR machine (Bio-Rad) for 60 min at 42°C and 5 min at 60°C. After running the protocol, cDNAs were stored at −20°C. GoTaq® Probe qPCR (Promega) was used for real time PCR and *18S* gene Cat. #4448490 (Thermo Fisher) was used as housekeeping gene. Plates containing Master Mix, genes of interest (LOX, LOXL1-2, Thermo Fisher) and housekeeping gene were loaded in RT-PCR ViiA™ 7 Real-Time PCR machine (Thermos Fisher Scientific). Cycling conditions were as follows: 2 min at 90°C, 40× (Denaturation 95°C for 15 s, Annealing/Extension 60°C for 60), dissociation 60–95°C. The relative abundance of RNA was calculated using the $2^{-\Delta\Delta Cq}$ method, and results were normalized to 18S RNA housekeeping gene.

2.8 | Animal tissues

All animal experiments were approved by the Animal Ethics Committee (DEC) of the University of Groningen and the Central Committee for Animal Experimentation (CCD) (AVD105002015303). Lung slices of 12-week-old C57BLJ6NTac mice (22–30 g), both male and female, were used for the experiments. Animals were housed in light and climate-controlled animal quarters with a temperature of 24°C and 12 h/12 h light-dark cycle and food and water ad libitum. Prior to preparation of lung slices, animals were euthanized by subcutaneous injection with ketamine (40 mg/kg) and dexdomitor (0.5 mg/kg) followed by exsanguination via the aorta abdominalis.

2.9 | Contraction

Murine lungs embedded with 1.5% agarose solution were cut in slices with a thickness of 250 μm according to published procedures.[23] The slices were incubated with 0.2 mM of the non-selective inhibitor of LOs activity β-aminopropionitrile (BAPN) (Sigma-Aldrich) or with Precision-Cut Lung Slices (PCLS) medium for 40 h at 37°C. Slices from 5 mice were included in the BAPN-treated group and slices from 10 mice in the control group. For the contraction experiment, one airway per slice was measured, and two slices per mouse were used. For the second harmonic generation (SHG) experiment, slices from three mice were assessed. Airways with a diameter between 300 and 500 μm were selected for the experiments. An increasing concentration of methacholine (MCh) (10^{-9} t/m 10^{-3} M) was added to the slices every 7.5 min and after the last concentration of MCh, the small airways were relaxed with chloroquine[24] (10^{-3} M). The airway narrowing was measured after each addition of the new concentration of MCh. Contraction of the airway was presented as a percentage of the basal area of the airway.

2.10 | Second harmonic generation

The microscope used in these experiments was a LSM Zeiss 780 NLO multiphoton confocal system coupled with a Chameleon Vision compact OPO two-photon laser. An excitation wavelength of 850 nm was used throughout. The microscope was equipped with 34-channel Quasar (32GaASP element and 2 sides PMT's) transmitted and reflected non-descanned light detectors. The objectives used were a 20× NA 0.8 and a 63× NA 1.4 objective. Emission was detected at 380–430 nm. Confocal images were taken at 8-bit resolution for backward-propagated SHG signals and for the autofluorescent signal of the tissue.

Image analyses were performed using Fiji[17] with images imported via the Bio-Formats plugin. The pixel area (area: the number of pixels with intensity above background) and pixel density (density: average signal intensity per pixel with intensity above background) were measures for each image. The above process was repeated for both the backward and the autofluorescent channel. The ratio of backward with the autofluorescent signal was then calculated for area. The average values for area and density were then calculated per group.

2.11 | Stiffness (elastic modulus) measurements

The stiffness property of the tissue was measured in terms of elastic modulus. The measurement was performed on the tissue immediately surrounding the airways in the slices from BAPN-treated group and control group using the micro-low-load compression tester (μLLCT). The samples were deformed by 20% of their original thickness (strain $\varepsilon = 0.2$) at a deformation speed of 20%/s (strain rate $\dot{\varepsilon} = 0.2/\mathrm{s}$) and the slope of the stress-stain curve was taken as the elastic modulus. For the elastic modulus test, slices of the BAPN-treated group and control group were obtained using the conditions as in the contraction section.

Slices were collected from five mice, and each mouse was treated separately to obtain the BAPN-treated group and control group. One site near an airway per slice was measured, and one slice for each group per mouse was used (more details about the μLLCT test are in Figure S1).

2.12 | Statistical analyses

Data analysis was performed using SPSS Statistics software, IBM (version 27). Data were initially tested for normality using the D'Agostino-Pearson omnibus normality test, and all data were expressed as median ± standard deviation. As IHC staining results included multiple measurements per patient, they were analyzed by a linear mixed effects regression model using SPSS. The effect of CSE exposure on LOs gene expression was analyzed using one-way analysis of variance, the results of the contraction study were tested using a Mann–Whitney *U* test, and the results of the elastic modulus test were tested using paired Student's *t*-test. α 0.05. $p < .05$ were considered significant.

3 | RESULTS

3.1 | *LOX* and *LOXL1*, but not *LOXL2*, gene expression levels were associated with COPD status, smoking status and lung function

Clinical characteristics of the 87 current and ex-smoking COPD patients and 151 controls present in the publicly

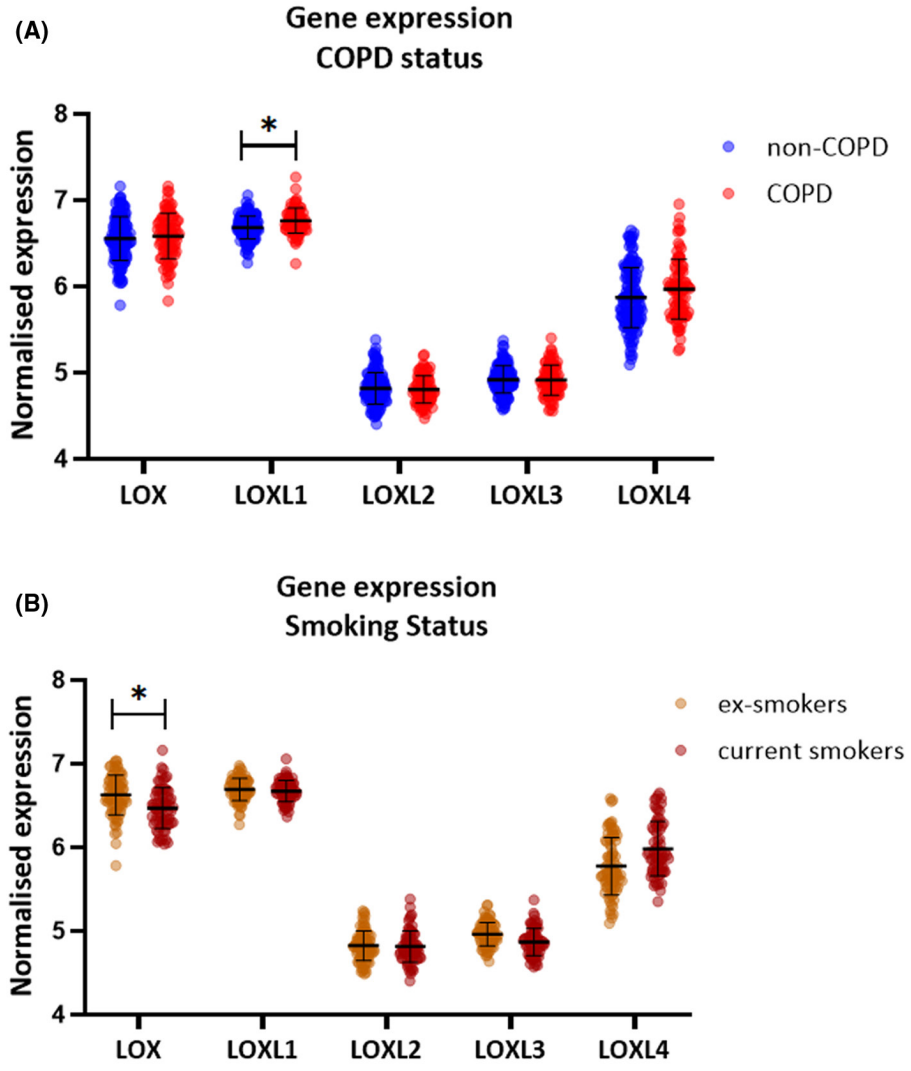

FIGURE 2 Lysyl oxidase gene expression comparing smoking and COPD status. Comparison of lysyl oxidase family of enzyme gene expression in bronchial brushes gene expression between COPD and non-COPD smokers (A) and ex-smokers and current smokers (B). Data were analyzed by using the linear model; *$p < .05$ LOX, lysyl oxidase; LOXL1-4, lysyl oxidase like-1-4.

available dataset (GSE37147) have been described previously.[16] No difference was present in age, smoke exposure and smoking status between COPD and control patients (Table 1). In bronchial brushes of COPD patients, there was a higher level of *LOXL1* ($p = 3.68 \times 10^{-5}$) gene expression compared to non-COPD ex-smokers and current smokers (Figure 2A and Table 2), and *LOXL1* gene expression was negatively correlated with FEV1% predicted ($p = .0143$). No such associations were observed for *LOX*, *LOXL2*, *LOXL3*, and *LOXL4*. When comparing current versus ex-smoking controls we observed that *LOX* gene expression was higher in current smokers ($p = 1.3 \times 10^{-7}$; Figure 2B). *LOXL1* and *LOXL2* gene expressions were not affected by smoking status (Table 2).

TABLE 2 Comparative analysis of the lysyl oxidase gene expression comparing COPD status, smoking status and association with lung function

Comparisons	Gene	*t*-value	*p*-value
COPD and non-COPD smokers	LOX	0.174	0.862
	LOXL1	4.208	3.68E-05*
	LOXL2	−0.616	0.538
Current and ex-smokers	LOX	5.446	1.16E-07*
	LOXL1	0.861	0.39
	LOXL2	−0.23	0.818
Association with FEV1% predicted	LOX	−1.111	0.268
	LOXL1	−2.467	0.0143**
	LOXL2	1.139	0.256

Note: Analysis performed using Robust Multichip Analysis (RMA) sketch algorithm workflow.

Abbreviations: LOX, lysyl oxidase; LOXL1, Lysyl oxidase like-1; COPD, chronic obstructive pulmonary disease; FEV1% predicted, forced expiratory volume in 1 s percentage predicted.

*Significant difference; **Significant correlation.

3.2 | Patient characteristics of IHC study

Parenchymal lung tissue samples containing small airways were obtained from 64 subjects. Of the 64 subjects, there were 3 control groups: non-COPD non-smokers, non-COPD ex-smokers and non-COPD current smokers ($n = 11$ per group). From now on, the non-COPD controls will be referred to as controls. The COPD groups consisted of subjects with mild COPD (GOLD stage II (18)) including 11 ex-smokers and 7 current smokers) and 13 ex-smoker patients with very severe COPD (GOLD stage IV) (Table 3). To investigate the effect of current smoking we assessed only the control groups and compared the combined non-smokers and ex-smokers to the current smokers. To investigate the effect of having COPD, the ex-smoking COPD stage II and COPD stage IV patients were compared to the ex-smoking controls. No significant differences were found between age and gender for all groups. Subject demographics are in Table 3.

3.3 | Expression patterns of LO family members in lung tissue

Immunohistochemistry staining was performed to determine LOs expression and localization in human small airways. Staining analyses showed that LOX, LOXL1, and LOXL2 proteins were constitutively expressed in the cytoplasm of epithelial cells and ASM (Figure 3A–C). LOXL3 protein was not detected in the lung tissue, whereas it was detected in colon, the positive control tissue. Weak LOXL4 staining was detected in the vessel walls, but not in small airways, epithelial layer or ASM and therefore was not further analyzed (Figure 3D).

TABLE 3 Patient demographics table

	Control never smokers	Control ex-smokers	control current smokers	COPD stage II ex-smokers	COPD stage II current smokers	COPD IV ex-smokers
n	11	11	11	11	7	13
Sex	7(f)/4(m)	7(f)/4(m)	3(f)/8(m)	8(f)/3(m)	4(f)/3(m)	6(f)/7(m)
Age (± SD) (years)	58.4 (11.6)	63.8 (8.6)	58.2 (7.5)	60.4 (10.4)	67.2 (12.2)	56.5 (4.0)
Height (± SD) (m)	1.69 (0.1)	1.72 (0.09)	1.71 (0.06)	1.69 (0.08)	1.73 (0.07)	1.7 (0.09)
FEV_1/FVC % (± SD)	79.1 (3.7)	78.1 (5.3)	75.9 (4.4)	58.5 (7.9)	58.0 (8.1)	24.6 (8.0)
FEV1% predicted (± SD)	96.4 (16.8)	97.3 (13.3)	96.3 (8.5)	70.9 (7.5)	61.2 (10.2)	19.6 (3.3)
FVC (L) pre BD (± SD)	3.6 (0.9)	3.8 (0.7)	4.0 (0.5)	3.7 (0.6)	3.4 (1.0)	2.8 (0.8)
GOLD stage	0	0	0	2	2	4

Note: Data presented as mean ± standard deviation (SD).

Abbreviations: BD, bronchodilator; f, female; FEV, forced expiratory volume; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, chronic obstructive lung disease; m, male.

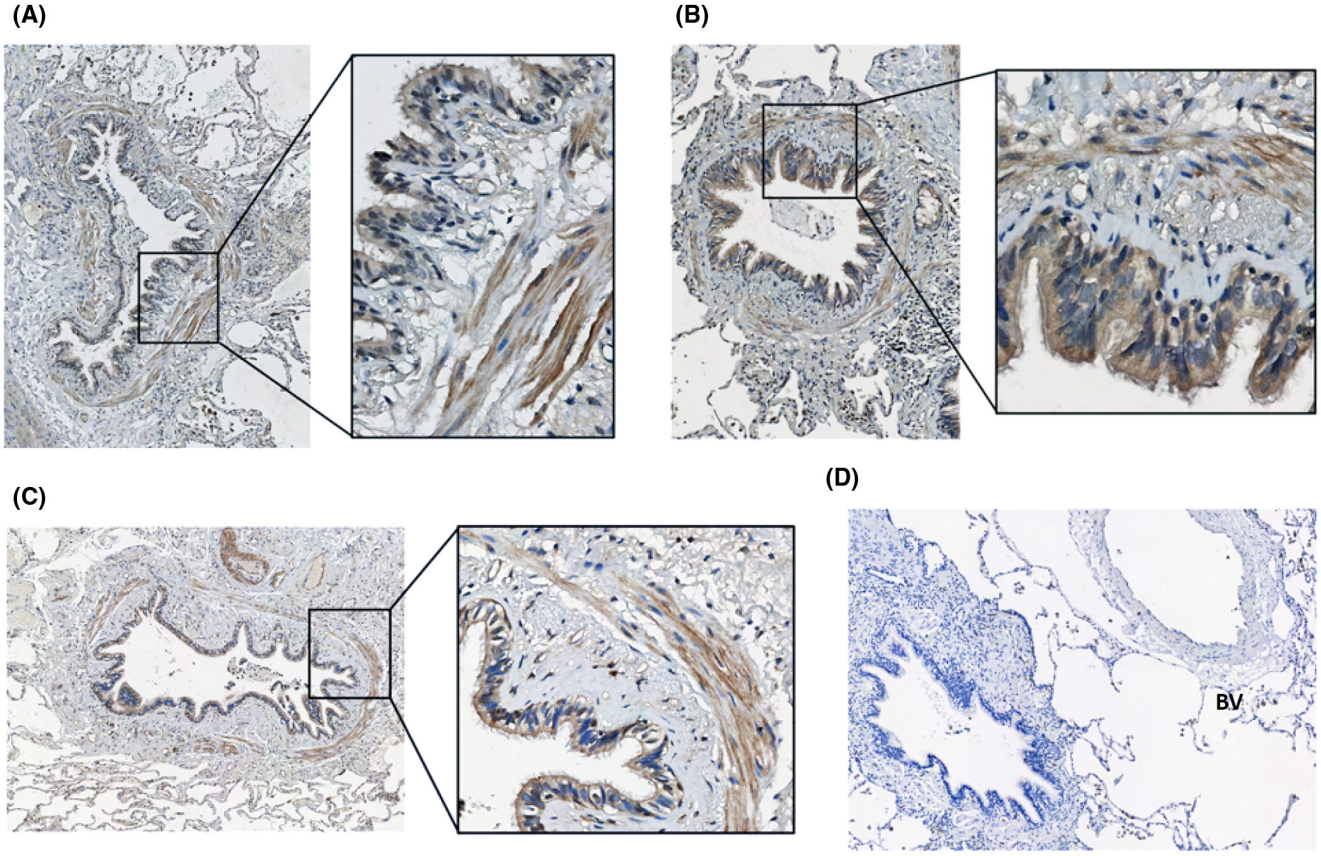

FIGURE 3 Representative expression of lysyl oxidase family of enzymes in small airways. LOX (A), LOXL1 (B), and LOXL2 (C) were constitutively expressed in the cytoplasm of epithelial cells and in airway smooth muscle, weak LOXL4 (D) staining was detected in the vessel wall but not in small airways. BV, blood vessel in the figures.

3.4 | Higher protein levels of LOXL1 and LOXL2 in current versus non-smoking controls

There was no effect of current smoking on LOX protein expression in small airways, epithelial layer, and ASM (Figure 4A–C). LOXL1 and LOXL2 protein expressions were higher ($p < .01$) in control current smokers compared to control non-smokers in all three regions of small airways: small airway as a whole, epithelial layer and ASM (Figure 4D–I).

3.5 | Differential protein expression of LO family members in COPD small airways

LOX protein expression was lower in the small airways and epithelial layer from COPD stage IV patients compared to control ex-smokers ($p < .01$) and COPD stage II patients ($p < .05$). COPD stage II patients were not different compared to control ex-smokers in the small airways and epithelial layer (Figure 5A,B). In addition, there was no difference in LOX protein expression between the controls, the COPD stage II and COPD stage IV patients in ASM (Figure 5C). COPD stage IV had higher LOXL1 protein expression in the epithelial layer compared to control ex-smokers ($p < .01$), and there was a trend towards higher LOXL1 protein levels in COPD stage II compared to control ex-smokers ($p = .11$) (Figure 5E). In the small airways and the ASM, LOXL1 level was not different in both COPD stage II and stage IV groups compared to the control group (Figure 5D,F). The LOXL2 protein level was not altered in COPD patients compared to control in all three regions of small airways: small airways as a whole, epithelial layer and ASM (Figure 5G–I) (Table 4).

3.6 | CSE upregulated *LOX* and *LOXL2*, but not *LOXL1*, gene expression levels in COPD ASM

LOs are differentially expressed in ASM of smokers compared to non-smokers. To determine the effect of smoking on LO gene expression in ASM, cells derived from non-/COPD patients were treated with CSE.

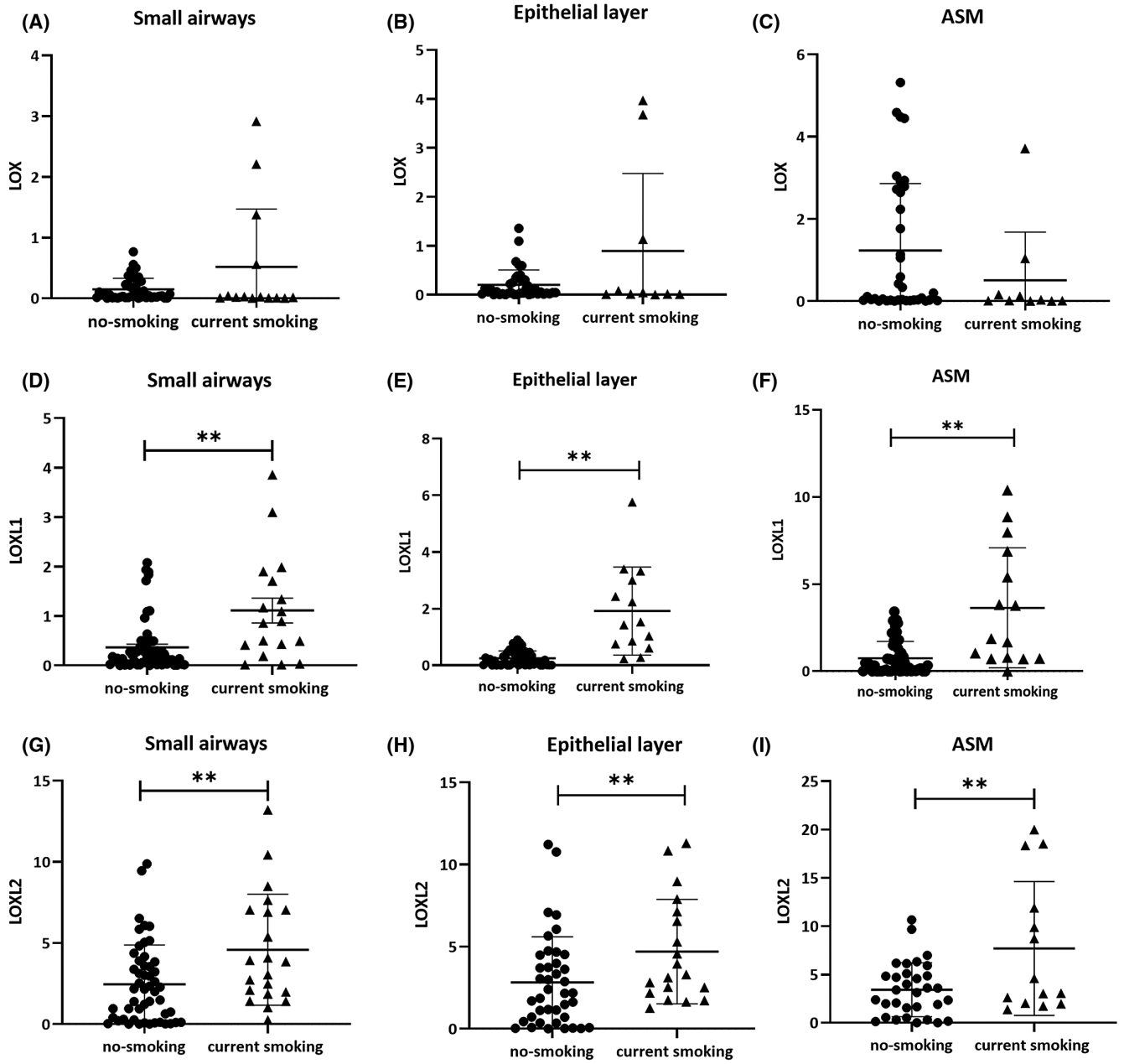

FIGURE 4 The effect of smoking on LOs protein expression. Image analysis results of LOX immunohistochemistry (IHC) staining in the airways (total) (A), epithelial layer (B), airway smooth muscles (ASMs) (C), LOXL1 IHC staining in small airways (total) (D), epithelial layer (E), ASM (F), and IHC staining of LOXL2 in small airways (total) (G), epithelial layer (H) and ASM (I). Data analyzed using linear mixed effects regression model; *$p < .05$; **$p < .01$. no-smoking, non-COPD never smokers and non-COPD ex-smokers, current smoking, non-COPD current-smokers; LOX, lysyl oxidase; LOXL1, lysyl oxidase like-1; LOXL2, lysyl oxidase like-2.

There was no difference observed in LO gene expression between untreated non- and COPD ASM (Figure S2A). COPD ASM cells treated with 10% CSE for 6 h had increased *LOX* and *LOXL2* gene expression compared to untreated cells; however, no difference was observed for *LOXL1* gene expression (Figure 6). Exposure to 1% and 5% CSE did not significantly affect LOs gene expression in COPD ASM. Stimulation of non-COPD ASM cells for 6 and 24 h and COPD cells for 24 h did not change LOX family gene expression (Figure S2B–D).

3.7 | The effect of LO inhibition on small airway contraction

BAPN is an irreversible inhibitor of LOs activity, which indirectly reduces formation of collagen cross-links and

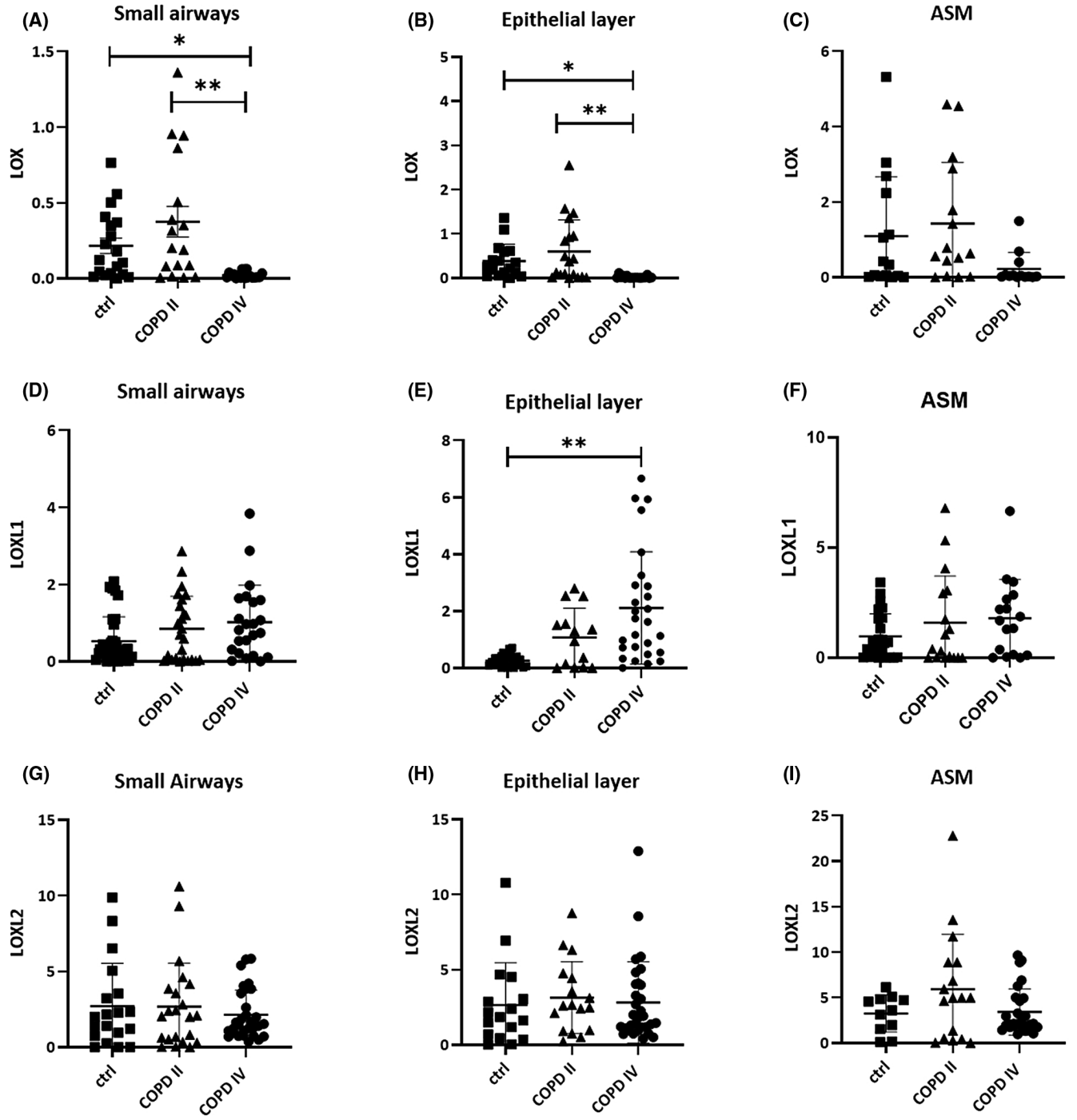

FIGURE 5 The effect of COPD on LOs protein expression. Image analysis results of LOX immunohistochemistry (IHC) staining in the airways (total) (A), epithelial layer (B), airway smooth muscle (ASM) (C), LOXL1 IHC staining in the airways (total) (D), epithelial layer (E), ASM (F) and IHC staining of LOXL2 in the airways (total) (G), epithelial layer (H), ASM (I). Data analyzed using linear mixed effects regression model; *$p < .05$. ctr, non-COPD ex-smokers; COPD II, ex-smokers COPD stage II; COPD IV, ex-smokers COPD stage IV; LOX, lysyl oxidase; LOXL1, Lysyl oxidase like-1; LOXL2, lysyl oxidase like-2.

maturation of collagen and therefore changes ECM biomechanical properties. To confirm the effect of BAPN on collagen crosslinking, SHG microscopy was performed on murine lung tissue treated with and without BAPN. The SHG signal was captured at the backward detector to provide information about the presence of disorganized/immature collagen.[4] In two out of the three mice examined, the BAPN group had a stronger backward signal compared to matched control slices, indicating an increase in disorganized/immature collagen in these tissues (Figure 7A). This is a promising observation that needs to be confirmed in larger studies. For the detection of

TABLE 4 Comparative analysis of the lysyl oxidase protein expression comparing smoking and COPD status

	Comparison	Protein	Small airways	Epithelial layer	ASM
Smoking	Control and smokers	LOX	–	–	–
		LOXL1	↑	↑	↑
		LOXL2	↑	↑	↑
COPD	Control and COPD stage II	LOX	↑	–	–
		LOXL1	–	–	–
		LOXL2	–	–	–
COPD	Control and COPD stage IV	LOX	↓	↓	–
		LOXL1	–	↑	–
		LOXL2	–	–	–

Abbreviations: "–", no difference; ASM, airway smooth muscle, in smoking status comparison control = non-smokers and ex-smokers; in COPD status comparison control = non-COPD ex-smokers; COPD, chronic obstructive pulmonary disease; LOX, lysyl oxidase; LOXL1, lysyl oxidase like-1.

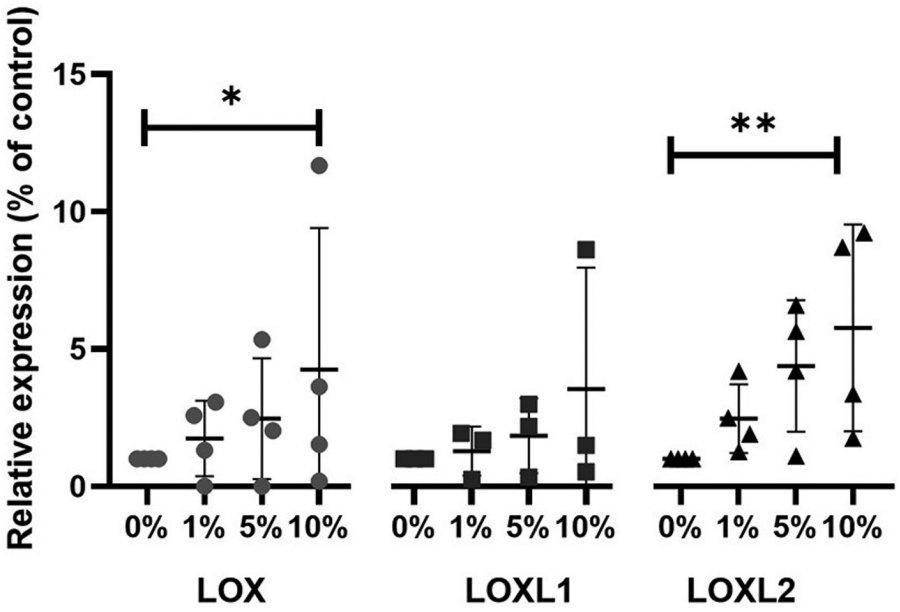

FIGURE 6 The effect of 6 h CSE exposure on LOs gene expression in COPD airway smooth muscle (ASM). Fold change of *LOX*, *LOXL1* and *LOXL2* gene expression of treated with CSE COPD ASM. Samples were stimulated with CSE 1%, 5% and 10% for 6 h. Data analyzed using one-way analysis of variance; *$p < .05$; **$p < .01$. ASM, airway smooth muscle cells; CSE, cigarette smoke extract; *LOX*, lysyl oxidase; *LOXL1*, lysyl oxidase like-1; *LOXL2*, lysyl oxidase like-2.

stiffness changes in slices, elastic modulus measurements on control and BAPN treated PCLS were performed using the μLLCT. The BAPN treated PCLS tissue around the airways had a lower stiffness compared to control PCLS ($p = .0284$, paired *t*-test) (Figure 7B), providing evidence that BAPN treatment could decrease the mechanical properties of the matrix surrounding the airways. To understand the role of LOs in small airway functions such as contraction, murine lung slices were incubated in the presence or absence of BAPN. Both control and BAPN treated PCLS contracted in response to MCh. BAPN treated slices showed greater contraction compared to the control group. Differences were achieved at four concentration points of MCh (10^{-7}, 10^{-5}, 10^{-4}, and 10^{-3} M). The relaxation curve induced by Cq was not different between the groups. For both groups – log of the concentration causing 50% effect (EC_{50}) was similar. Maximal contraction E_{max} was greater in the BAPN treated group (26% of remaining area of lumen open) compared to 47% in a control group (Figure 7C).

4 | DISCUSSION

To our knowledge, this is the first study to examine the expression of the LO enzymes family in the airways in

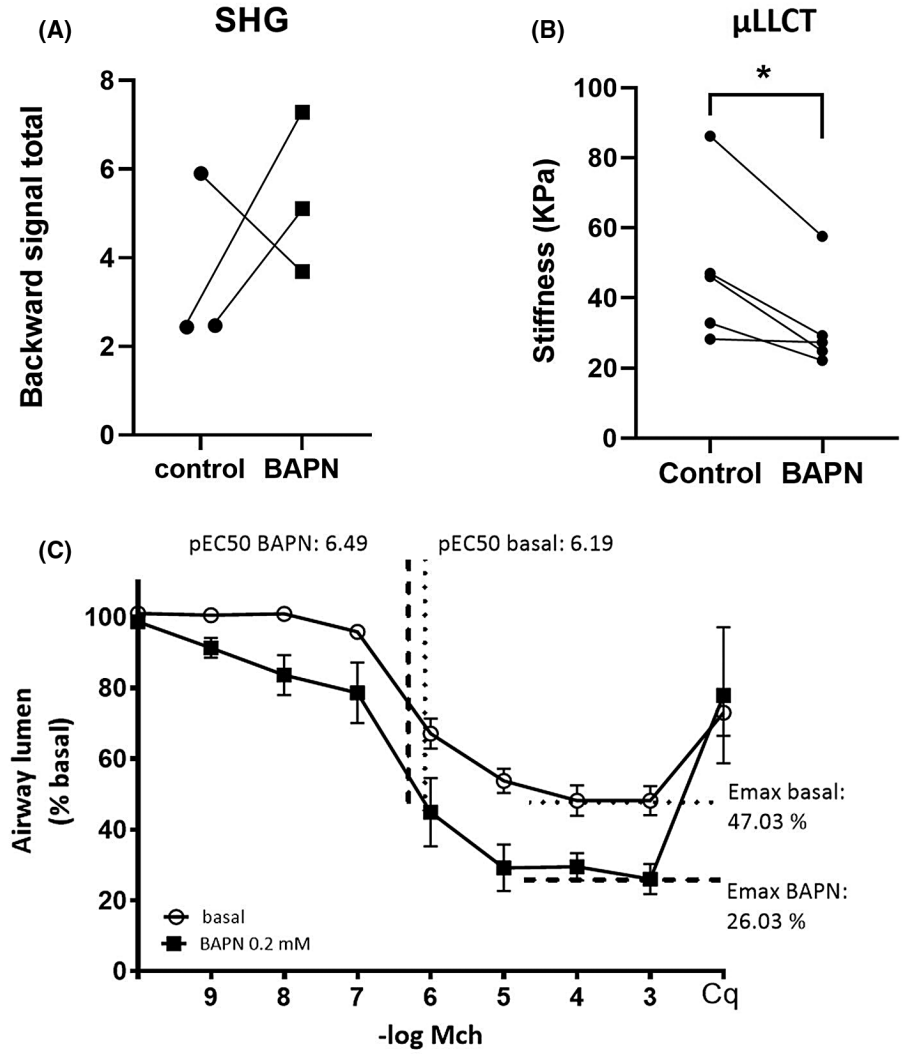

FIGURE 7 The effect of BAPN on matrix organization and airway responsiveness. Increased level of collagen disorganization/immaturity in BAPN treated lungs; image analysis quantification of second harmonic generation (SHG) intensity backward signal in control and BAPN-treated murine lung slices ($n = 3$) (A), comparison of stiffness of control and BAPN treated lung slices; slices were mechanically tested by using micro-low-load compression tester (µLLCT) at 20% strain. Each dot represents one measurement per slice for each mouse ($n = 5$) (B), airway responsiveness toward dose dependent methacholine (MCh) treatment of small airways in lung slices obtained from mice. Values are means of 5 mice (2 slices per mice) in BAPN treated group and 10 mice (2 slices per mice) in control group (C), *$p < .05$. BAPN, B-aminoproprionitrile; MCh, methacholine; Cq, chloroquine; pEC_{50}, concentration causing 50% effect; E_{max}, maximal constriction. The results were tested using a Mann–Whitney *U* test (A,C) or paired Student's *t*-test (B). $p < .05$ were considered significant.

relation to the presence and severity of COPD and in relation to smoking status. Gene expression analysis in bronchial brushing showed that expression of *LOX* was higher in smokers compared to ex-smokers and *LOXL1* was higher in COPD compared to non-COPD. In vitro analyzes illustrated that COPD ASM cells were more sensitive to CSE exposure compared to non-COPD cells and expression of *LOX* and *LOXL2* were upregulated in COPD ASM cells after exposure to CSE compared to untreated COPD cells. LOXL1 and LOXL2 protein expression was clearly elevated in small airways derived from current smokers compared to ex-smokers, and, increased gene and protein expression of LOXL1 was detected in COPD airways compared to non-COPD. LOX protein expression was lower in COPD small airways compared to the non-COPD group, but no differences were observed for LOXL2 protein expression in COPD. Therefore, our findings indicate differential regulation of LO family members in the airways of current smokers and COPD patients, with LOXL1 being the only member showing consistent higher levels with both smoking and COPD. As LOXL3 protein expression was not detected in the lungs, and weak LOXL4 protein staining was mainly found in the vessels and not in the airways, it is unlikely that LOXL3 and LOXL4 play an important role in collagen organization in the small airways.

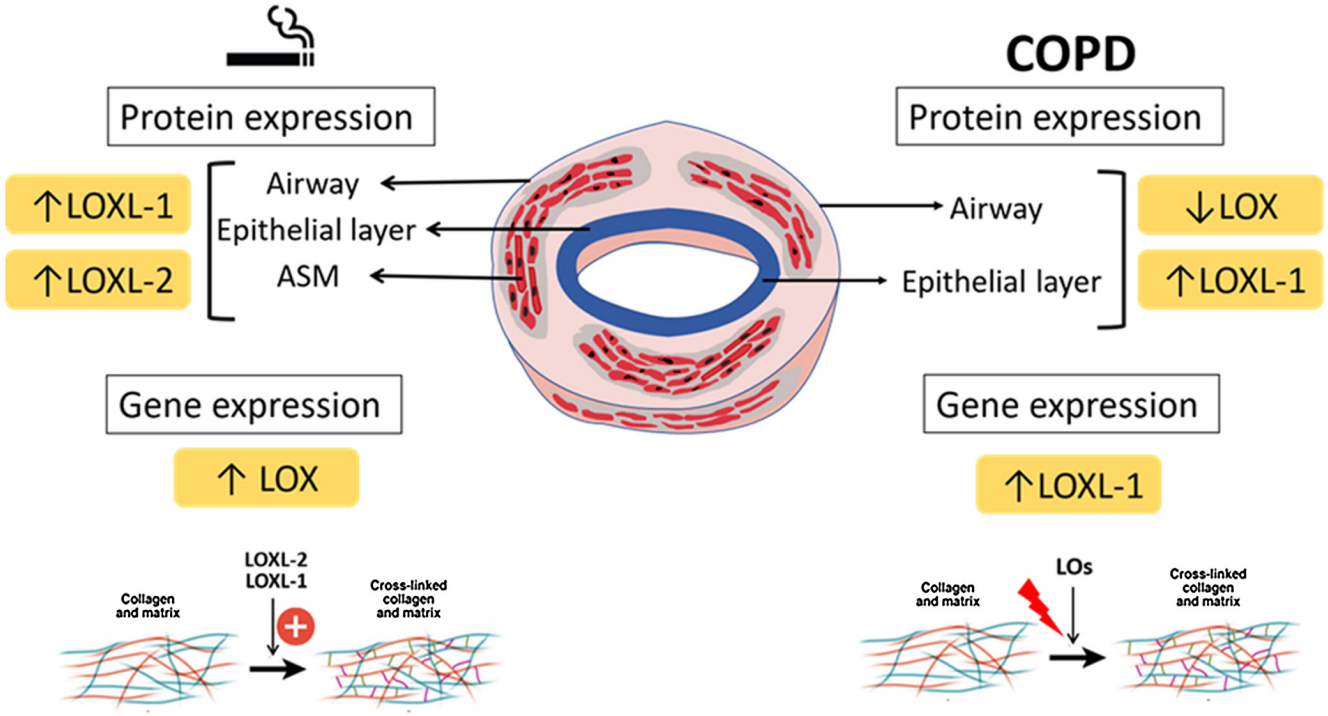

FIGURE 8 Schema of the effect of smoking and COPD on LOs expression in small airways and alteration of collagen cross-linking. LOX, lysyl oxidase; LOXL1, lysyl oxidase like-1; LOXL2, lysyl oxidase like-2; ASM, airway smooth muscle.

Changes of LOs expression induced by smoking can be one of the factors contributing to development of COPD. In this study, we observed that smokers without COPD had increased *LOX* gene expression and LOXL1 and LOXL2 protein expression in the airways suggesting that in the small airways of current smokers LOs protein and gene expression are constantly upregulated in response to active smoking. In COPD ASM cells exposure to smoke increased *LOX* and *LOXL2* gene expression. As LOs are known to induce fibrosis and also have the ability to alter the ECM to form a pathological environment,[25] this constant upregulation may contribute to dysregulated collagen crosslinking in the airway and pose a risk factor for the development of small airway remodeling in COPD or aggravate already existing changes in COPD. In rat fetal lung fibroblasts LOs expression have previously been reported to be suppressed following cigarette smoke exposure at multiple levels including mRNA, protein and catalytic activity,[26,27] which is different from our observations. This may be explained by the difference in lung compartment, cell type, developmental stage and the model that was studied, ie we studied ex vivo gene expression in epithelial brushes and protein expression in small airways in humans, compared to the in vitro fetal fibroblasts in the rat model.

Overall, the findings in our study show that LOs family members respond in dissimilar ways to smoking stimuli and are differentially expressed in COPD airways, indicating that different LOs may have diverse functions in the lung. Interestingly, COPD did not influence protein expression of LOs in ASM, suggesting that ASM cells are not involved in LOs induced changes in COPD airways.

Lower expression of LOX and higher expression of LOXL1 levels possibly suggest a role for these enzymes as regulatory factors for pathogenic cross-linking of collagen in the small airways in COPD. This could indicate a differential role for LOX and LOXL1 in the pathology of COPD. LOXL1 protein levels were higher in all compartments of small airways of smokers without COPD compared to nonsmokers and in the epithelial layer of COPD patients. This could indicate that changes of LOXL1 expression induced by smoking can be an important contributor to the development of COPD in a subset of susceptible individuals and it may play a role in the occurrence of fibrosis in the small airways. LOXL2 protein expression was clearly elevated in current smokers without COPD but not in COPD patients. Taken together, LOX may be the enzyme that regulates the fundamental collagen cross-linking in the small airways, whereas LOXL2 appears to be the enzyme that responds to external stimuli to regulate collagen cross-linking, and LOXL1 can be involved in both functions.

A precedent for differential expression of LOX and LOXL1 is seen in the rat aorta during growth and aging[28] and also differential expression of LOs has been associated with distinct pathological processes such as tumor progression and metastasis.[29,30] In 2017, Tjin et al. showed that increased LOXL1 played a role

in collagen remodeling in IPF lungs, where there were decreased levels of LOX.[12] These data are in concert with our results. Gilead Sciences tested the humanized monoclonal antibody against LOXL2 (simtuzumab) for the treatment of patients with IPF. However, in 2016, the phase 2 clinical trial was terminated due to its lack of efficacy.[31] Together with the results illustrating LOs protein expression in COPD and IPF lungs we can postulate diverse contributions of the individual LO family members in disease progression. This brings us to the idea that a possible reason for the inefficiency of simtuzumab therapy could be the targeting of only one LO family member, whereas effective treatment may require targeting of several LOs proteins at the same time. In COPD, the fibrosis and obstruction of the small airways have been reported, exhibiting a strong correlation with the disease severity.[32,33] The decrease of LOXL1 activity and stimulation of LOX in COPD airways may normalize the turnover of collagen cross-linking in the fibrotic small airways leading to stabilization of small airways biomechanical functions. Therefore, modulation of both LOX and LOXL1 may provide novel targets for the treatment of airway fibrosis in COPD (Figure 8, Table 4). The availability of selective modulators of LOs would greatly improve understanding of a role of LOs in various pathological processes. Some compounds have been developed as inhibitors with dual activity against both LOX and LOXL2,[34] but there is a lack of specific inhibitors for some members of the LOX family. Currently the lack of commercially available selective inhibitors and blockers is a limitation in the field.

LOs are important players in the regulation of the tissue biomechanical environment by mediating collagen cross-linking and promoting maturation and organization of collagen fibrils.[35] Changes in LOs expression and activity can cause collagen structural remodeling in both parenchyma and small airways that can both affect the lung function. In this study, we only focused on the role of LOs in the airways, additional studies would be needed to address the role of these enzymes in the parenchyma as well, especially since previous research showed that a decrease of LOX, LOXL1, and LOXL2 expression had been associated with emphysematous alteration in COPD.[36] To demonstrate the role of LOs activity in airway mechanics, murine lung slices were treated with a non-selective inhibitor of LOs activity BAPN. BAPN is commonly used in various pre-clinical studies, and it has demonstrated interesting results in murine cancer models.[34] However, lack of sites for chemical modification and high toxicity makes this molecule unsuitable for clinical trials.[37]

BAPN treated slices showed greater contraction than untreated slices, which is possibly due to the loss of collagen cross-linking. Also, detection of elastic modulus using μLLCT illustrated decreased stiffness of ECM surrounding the airways in the BAPN treated group. The loss of collagen cross-linking is known to result in the softening of the tissue surrounding the ASM, and as a consequence, this tissue becomes more responsive to the forces generated by the ASM.[38] As ECM is a part of the surrounding tissue, and a key player in the connection of the parenchymal compartment and the small airways, disruption of collagen cross-link formation in parenchymal ECM can lead to the reduction of parenchymal tethering forces that counteract the force of ASM contraction. Therefore, loss of collagen cross-links can lead to greater contraction in small airways.[24,38]

In conclusion, this study is the first to explore the role of the LOs family members in relation to presence and severity of COPD and smoking status, covering changes in the gene expression and protein levels as well as functional studies on airway contraction. These findings highlight the potential differential roles of LOs enzymes in the lung and clarify the need to focus on the role of each protein separately for further elucidation of their individual contributions to the pathogenesis of COPD in small airways and parenchyma.

AUTHOR CONTRIBUTIONS

Janette K. Burgess, Corry-Anke Brandsma, Wim Timens, Reinoud Gosens and Prashant K. Sharma conceived the study and designed the research; Nataliya Migulina, Gavin Tjin, Marit Metzlar, Theo Borghuis, Fenghua Zhao and E. van Dijk performed research; N. Migulina, G. Tijn, A. Faiz, M. Metzlar and Eline van Dijk analyzed data; Alen Faiz, Theo Borghuis and Hans J. Kaper contributed analytical tools; Nataliya Migulina, Janette K. Burgess and Corry-Anke Brandsma drafted the paper; and all authors edited and approved the final version of the manuscript.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support gene expression analysis in bronchial brushing are publicly available from affymetrix_Hu_gene_st1.0 arrays from (GSE37147) at https://doi.org/10.1164/rccm.201208-1449OC. The data that support the findings of LOs protein expression in human tissue, gene expression in ASM cells, and contraction, second harmonic generation and stiffness (elastic modulus) measurements in animal tissue are available on reasonable request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Nataliya Migulina https://orcid.org/0000-0002-3884-7763

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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