ORIGINAL ARTICLE

Relationship between the thickness of bronchial wall layers, emphysema score, and markers of remodeling in bronchoalveolar lavage fluid in patients with chronic obstructive pulmonary disease

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KEY WORDS

chronic obstructive pulmonary disease, computed tomography, emphysema score, endobronchial ultrasound, markers of remodelling

ABSTRACT

INTRODUCTION Airway remodeling plays an important role in the development of chronic obstructive pulmonary disease (COPD). Imaging methods, such as computed tomography (CT) and endobronchial ultrasound (EBUS), may be useful in the assessment of structural alterations in the lungs.

OBJECTIVES The aim of this study was to evaluate a relationship between the severity of emphysema assessed by chest CT, the thickness of bronchial wall layers measured by EBUS, and the markers of remodeling in bronchoalveolar lavage fluid (BALF) in patients with COPD.

PATIENTS AND METHODS The study included 33 patients with COPD who underwent pulmonary function tests, emphysema score assessment by chest CT, as well as bronchofiberoscopy with EBUS in order to measure the total bronchial wall thickness and, separately, layers L_1, L_2, and $L_{3–5}$. Selected remodeling (matrix metalloproteinase 9 [MMP-9], tissue inhibitor of metalloproteinase 1, transforming growth factor β_1 [TGF-β_1]) and inflammatory markers (neutrophil elastase, eosinophil cationic protein) were measured in BALF samples using an enzyme-linked immunosorbent assay.

RESULTS MMP-9 levels in BALF were significantly higher in patients with very severe bronchial obstruction than in those with moderate and mild bronchial obstruction ($P = 0.02$), and showed a negative correlation with forced expiratory volume in 1 second ($r = –0.538$, $P = 0.002$). The thickness of L_1 and L_2, which histologically correspond to the mucosa, submucosa, and smooth muscle, demonstrated a positive correlation with TGF-β_1 levels in BALF ($r = 0.366$, $P = 0.046$ and $r = 0.425$, $P = 0.02$) and the thickness of L_1 showed a negative association with neutrophil elastase levels ($r = –0.508$, $P = 0.004$). There was no significant correlation between the analyzed markers in BALF and the emphysema score.

CONCLUSIONS Significant correlations of TGF-β_1 and elastase with the thickness of bronchial wall layers, and of MMP-9 with the severity of obstruction, may suggest the involvement of these markers in airway remodeling in patients with COPD.

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INTRODUCTION Chronic obstructive pulmonary disease (COPD) is characterized by progressive, only partially reversible airflow limitation secondary to resistance of the small conducting airways and emphysematous destruction of the lungs.[1] Inhalation of smoke particles and noxious gases triggers the inflow of multiple cells, especially neutrophils and macrophages, secreting a number of proinflammatory substances.[2,3] Chronic inflammation leads to structural alterations in the airways, known as "airway remodeling".[4] Hypertrophy and hyperplasia of goblet cells and submucosal glands result in an increased production of mucus and impaired mucociliary clearance.[5,6] Oxidizing agents contained in tobacco smoke play an important role in the destruction of lung parenchyma, as they cause protease–antiprotease imbalance, resulting in the loss of elastic fibers and, consequently, in emphysema.[3]

Lung function tests play a crucial role in the diagnosis and monitoring of COPD. Forced expiratory volume in 1 second (FEV_1) is the most widely used parameter in the assessment of disease severity and progression. However, this method does not allow identification of the cause of airflow limitation or determination of remodeling severity.[7] Unfortunately, despite intensive research, no other optimal methods of COPD evaluation have been found yet.

Imaging methods are very useful tools for assessing the severity of remodeling. Endobronchial ultrasound (EBUS) enables the measurement of total bronchial wall thickness and distinguishing its particular layers.[8] Computed tomography (CT) of the chest not only allows an estimation of bronchial wall thickness, but also enables a quantitative evaluation of the severity of emphysema by measuring the emphysema score.[9] Potential remodeling markers in serum and bronchoalveolar lavage fluid (BALF) may correlate better than spirometry with activity of disease and severity of morphological changes in the airways.[10,11]

There are only a few studies in patients with COPD that explored the relationship between markers of remodeling in BALF and structural changes in the lung assessed by CT imaging.[12-15] In our previous study, we showed the utility of EBUS in the assessment of the bronchial wall thickness in COPD patients.[8] However, little is known whether there is any association between locally produced mediators and bronchial wall thickness. Therefore, the aim of this study was to evaluate the relationship between markers of airway remodeling in BALF and the thickness of bronchial wall layers measured by EBUS.

PATIENTS AND METHODS **Characteristics of patients and study design** Thirty-three patients with stable COPD were enrolled between September 2010 and July 2013 at the Department of Pulmonology of University Hospital, Kraków, Poland (9 women, 24 men; mean age, 66.8 ±8.8 years). The severity of COPD was evaluated according to the Global Initiative for Chronic Obstructive Lung Disease guidelines.[16] The median value of disease duration was 6.0 years (lower and upper quartiles, 2.8–10 years) and of pack years—39 (lower and upper quartiles, 25–60). Thirty patients were taking inhaled corticosteroids, and the mean daily dose of fluticasone was 738.3 ±397.9 μg. Additionally, 11 patients were taking oral corticosteroids (the mean daily dose of methylprednisolone was 8.4 ±4.2 mg). Data are presented in **TABLE 1**. The exclusion criteria included a history of any other chronic pulmonary disease and any conditions that are a contraindication to perform pulmonary function tests, bronchoscopy, or CT. The study was approved by the Ethics Committee of Jagiellonian University Medical College, Kraków, Poland, and written informed consent was obtained from all participants.

TABLE 1 Summary of demographical characteristics and the results of lung function tests and biomarker analysis in the study population

Parameter	Mean ±SD or median (25%–75%)
age, y	66.8 ±8.8
FEV_1, percentage of predicted value	57.8 ±22.3
duration of the disease, y	6.0 (2.8–10)
smoking, pack-years	39 (25–60)
emphysema score, percentage of lung tissue below –910 HU	31.2 (10.9–46.2)
MMP-9, ng/ml	6.7 (2.1–26.1)
TIMP-1, ng/ml	5.0 (3.6–7.3)
TGF-β_1, ng/ml	8.0 (4.6–15.3)
neutrophil elastase, ng/ml	2.1 (1.0–7.1)

Abbreviations: FEV_1, forced expiratory volume in 1 second; HU, Hounsfield unit; MMP-9, matrix metalloproteinase 9; TGF-β_1, transforming growth factor β_1; TIMP-1, tissue inhibitor of metalloproteinase 1

Spirometry was performed (Jaeger MasterLab, Höchberg, Germany) before and after administration of short-acting β_2-agonist in order to assess bronchial reversibility. Additionally, bodyplethysmography (Jaeger Master Screen PFT, Höchberg, Germany) was performed to measure total lung capacity (TLC) and residual volume (RV).

Lung computed tomography and emphysema-score assessment Chest CT was performed using 64-row multidetector computed tomography (Aquilion TSX-101A, Toshiba Medical Systems Corporation, Otawara, Japan) in a helical scanning mode (CT parameters: 64 × 0.5 mm collimation, helical pitch of 53 and 0.5 second per rotation with standard radiation dose [150 ±50 mAs and 120 kVp]) and without administration of intravenous contrast medium. We used the emphysema score to assess the level of emphysema seen in CT scans. Emphysema is characterized by a decrease in lung tissue mass and blood vessel area and an increase in the areas of trapped air, causing an overall decrease in

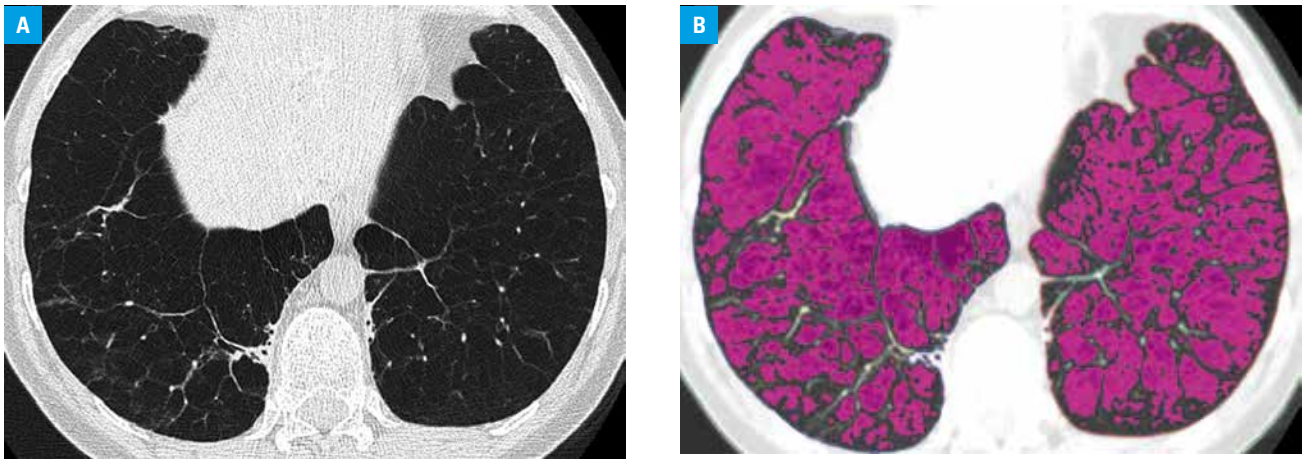


FIGURE 1 **A** – Chest computed tomography of patients with chronic obstructive pulmonary disease showing advanced emphysema as an area of decreased attenuation; **B** – measurement of the emphysema score using the Pulmo-CMS software (areas of decreased attenuation are shown in pink color).

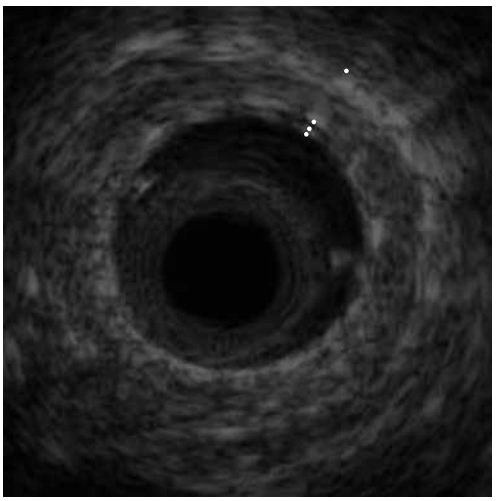


FIGURE 2 Endobronchial ultrasound image of the patient with chronic obstructive pulmonary disease. The first layer (from the first to second points) at the luminal side corresponds to the mucosa and inner part of the submucosa. The second layer (from the second to third points) is the outer part of submucosal tissue, which includes smooth muscles. Layers 3 to 5 (the distance between the third and fourth points) correspond to cartilage.

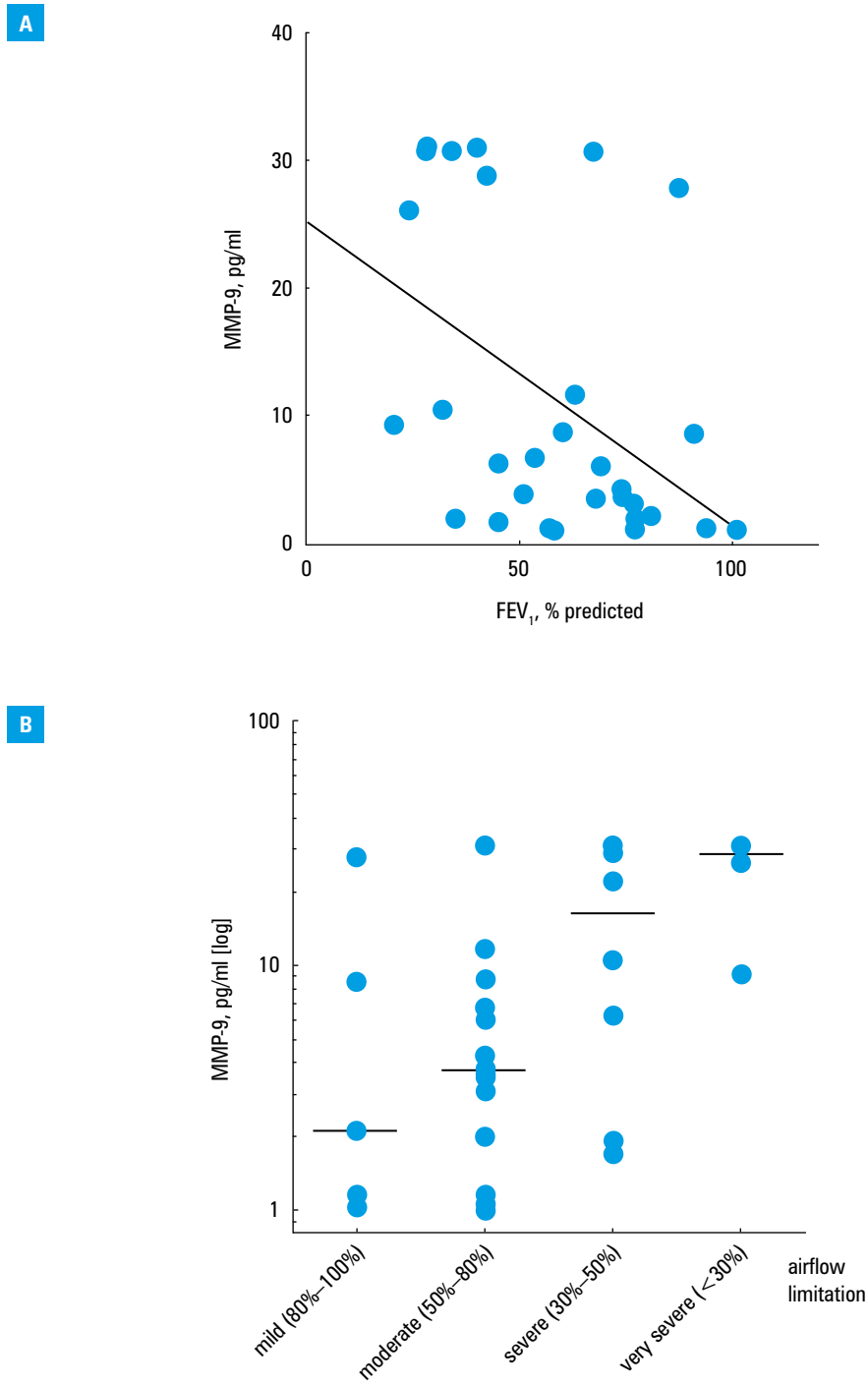
lung density. The emphysema score was quantified by the Pulmo-CMS software (Medis specials, Leiden, the Netherlands) and was defined as the percentage of lung tissue below the designated attenuation threshold value of –910 Hounsfield units. This index determines the percentile quantity of voxels showing a value lower than preset attenuation (in Hounsfield units) from all voxels within lung tissue (FIGURES 1A and 1B). We analyzed CT scans of 1-mm images obtained with a soft tissue reconstruction algorithm. The analysis of CT scans was carried out by one independent radiologist.

Bronchoscopy and endobronchial ultrasound Bronchofiberoscopy was carried out under local anesthesia (lidocaine, 2%) and mild sedation with fentanyl (0.05 to 0.1 mg IV) and midazolam (2.5 to 5 mg IV). Endobronchial ultrasonography was performed with a BF1T180 bronchofiberoscope (Olympus; Tokyo, Japan), a 20-MHz ultrasonographic probe, and a processor (EU-ME1, Olympus). The probe was placed in the posterior basal bronchus of the right lower lobe (RB10). Our method allowed us to discriminate 5 layers in the airway wall that correlated with the laminar histological structures of the bronchi. The first layer (L_1) at the luminal side corresponds to the mucosa and inner part of the submucosa, and the second layer is the outer part of submucosal tissue, which includes smooth muscles. The outer layers (layers 3 to 5 [$L_{3–5}$]) correspond to cartilage and were analyzed jointly (FIGURE 2).[8,17,18] Digital images recorded during EBUS (movie frames) were analyzed using a dedicated software converting data from the raster to vector format with subpixel precision (Feature Extraction Software, FES, AGH, Kraków, Poland). The FES software enabled us to measure the distance between 2 points in the image representing the borders of particular airway wall layers (marked manually). Measurements of each layer (L_1, L_2, $L_{3–5}$) were performed in 5 representative images, and the mean values were treated as the final result.

Immune mediators in bronchoalveolar lavage fluid A bronchofiberoscope was wedged in the segmental bronchus of the right middle lobe, and 4 portions of sterile 0.9% saline solution (200 ml in total) were instilled. Aliquotes of BALF samples were collected and stored at –80°C for further measurements. The concentrations of human tissue inhibitor of metalloproteinase 1 (TIMP-1), transforming growth factor β_1 (TGF-β_1), and human matrix metalloproteinase 9 (MMP-9) were measured by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minnesota, United States) according to the manufacturer's instructions. The levels of human neutrophil elastase (NE) were measured by an ELISA (Platinum ELISA, eBioscience, San Diego, California, United States) and the concentration of eosinophil cationic protein (ECP) was assessed using ImmunoCAP assays (Phadia, Uppsala, Sweden).

Statistical analysis All calculations were performed with STATISTICA 12 data analysis software system(StatSoft, Inc. Tulsa, Oklahoma, United States). Categorical variables were

FIGURE 3 Correlation between matrix metalloproteinase 9 (MMP-9) levels in bronchoalveolar lavage fluid (BALF) and forced expiratory volume in 1 second (FEV_1) (**A**); MMP-9 levels in BALF according to the severity of obstruction (**B**)



presented as numbers and percentages. Continuous variables were expressed as mean ± SD or median and lower and upper quartiles, as appropriate. The study group was divided into 4 groups according to the severity of obstruction. The comparisons of the mean levels of studied markers between these groups were performed using a general linear model (after variance stabilizing transformation when needed). Correlations between variables were estimated with the Spearman rank order correlation coefficient. A *P* value of 0.05 or less was considered statistically significant.

RESULTS Altogether, 33 patients with COPD, staged on the basis of FEV_1 results as mild (n = 5), moderate (n = 13), severe (n = 11), and very severe (n = 4), were included in the final analysis. The mean FEV_1 was 57.8% ±22.3%. FEV_1 in liters (r = –0.531, P = 0.004) and as a predicted value (r = –0.55, P = 0.002) were negatively correlated with the duration of the disease. Moreover, disease duration showed a positive correlation with NE concentrations (r = 0.437, P = 0.02). The number of pack-years showed a negative correlation with the concentration of TIMP-1 in BALF (r = –0.431, P = 0.045).

The mean total bronchial wall thickness assessed by EBUS was 1.192 ±0.079 mm. The thickness of the first bronchial wall layer (L_1) was 0.135 ±0.018 mm; the second (L_2), 0.151 ±0.026 mm; and the last (L_{3-5}), 0.906 ±0.065 mm. There was no association between bronchial wall thickness and parameters measured by pulmonary function tests.

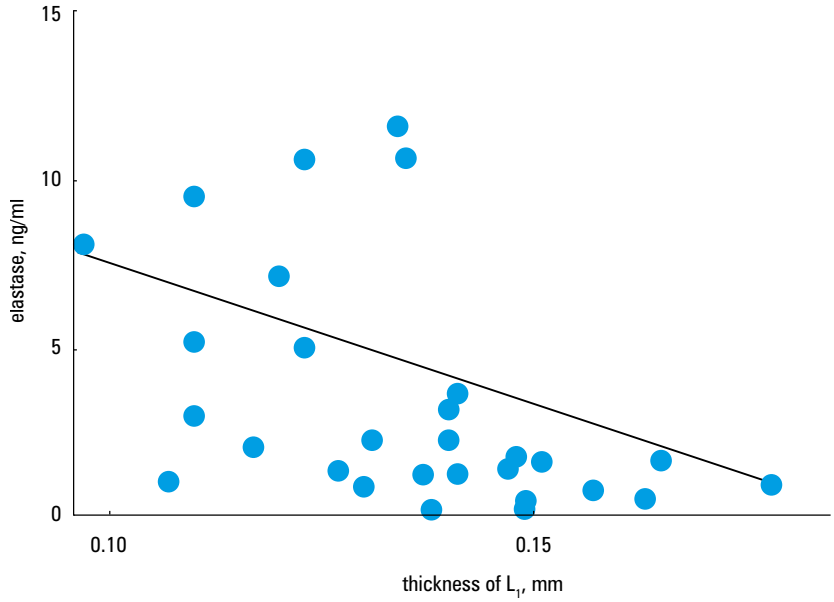


FIGURE 4 Correlation between the thickness of layer 1 (L_1) of the bronchial wall and the concentration of neutrophil elastase in bronchoalveolar lavage fluid

There was no significant correlation between the emphysema score and total bronchial wall thickness or its individual layers. The median value of the emphysema score in COPD patients was 31.2% (lower and upper quartiles, 10.9%–46.2%). There was no correlation between the emphysema score and FEV_1, TLC, RV, or RV/TLC. Moreover, there was no association between the emphysema score and the concentration of NE, MMP-9, TIMP-1, ECP, and TGF-β_1.

The concentration of MMP-9 was negatively correlated with FEV_1 (r = –0.538, P = 0.002). Moreover, it was higher in patients with very severe obstruction than in patients with moderate or mild obstruction (P = 0.02) (FIGURE 3A and 3B). There was no correlation between FEV_1 and the other markers of remodeling.

The thickness of L_1 showed a negative correlation with the concentration of NE in BALF (r = –0.508, P = 0.004) (FIGURE 4), and the thickness of L_1 and L_2 demonstrated a positive correlation with the concentration of TGF-β_1 in BALF (r = 0.366, P = 0.046 and r = 0.425, P = 0.02) (FIGURE 5). There was no correlation between the total thickness of the bronchial wall and its layers and the concentrations of MMP-9, TIMP-1, and ECP. The results showed that there was no association between the parameters measured by imaging methods and concomitant eosinophilic infiltration.

DISCUSSION An imbalance between degradation and deposition of extracellular matrix components is responsible for increasing resistance in small bronchi and bronchioles and increasing lung compliance due to emphysema. In the literature, there are numerous publications on the potential markers of remodeling in COPD,[12-15] but there are no data assessing the relationship of these markers and bronchial wall thickness determined by EBUS. To our knowledge, this is the first study to examine the relationship between remodeling markers in BALF and bronchial wall thickness assessed using EBUS in patients with COPD.

TGF-β_1 is one of the most important growth factors involved in the pathogenesis of COPD. Activation of the TGF-β_1 signaling pathway causes airway fibrosis and development of emphysema, mainly by affecting the production and degradation of collagen in the extracellular matrix.[2] In our study, we found a significant positive correlation between the concentration of TGF-β_1 in BALF and the thickness of the first and second layers of the airway wall (L_1, L_2), which corresponds to the mucosa, submucosa, and smooth muscle. In COPD, an increased expression of TGF-β_1 in the airway epithelium has been associated with enhanced fibrotic airway remodeling.[5] For example, in a study by Vignola et al,[19] an increased expression of TGF-β_1 correlated with the number of peribronchial fibroblasts and the thickness of the basement membrane. Using the rodent model, Kenyon et al[20] demonstrated that intratracheal administration of recombinant TGF-β_1 leads to increased deposition of collagen and thickening of the basement membrane in small airways. However, the impact of TGF-β_1 signaling on the pathogenesis of emphysema seems to be less well defined. In the present study, we also found no association between the concentration of TGF-β_1 in BALF and the emphysema score. Similarly, Stoll et al[21] showed no relationship between the level of TGF-β_1 in serum and severity of emphysema as measured by carbon monoxide diffusing capacity and residual volume.

The reduction of TGF-β_1 secretion may cause an increased expression of matrix metalloproteinases (MMPs), which leads to emphysema resulting from degradation of the extracellular matrix. MMP-9 activates signaling pathway of TGF-β_1, which contributes to increased fibrosis of the airways.[22] Tissue metalloproteinase inhibitors prevent destructive activity of MMPs. Gelatinase B (MMP-9) is responsible for degradation of collagen IV, a major component of the basement membrane, and elastin fibers.[23-26] In our study, the concentration of MMP-9 was higher in patients

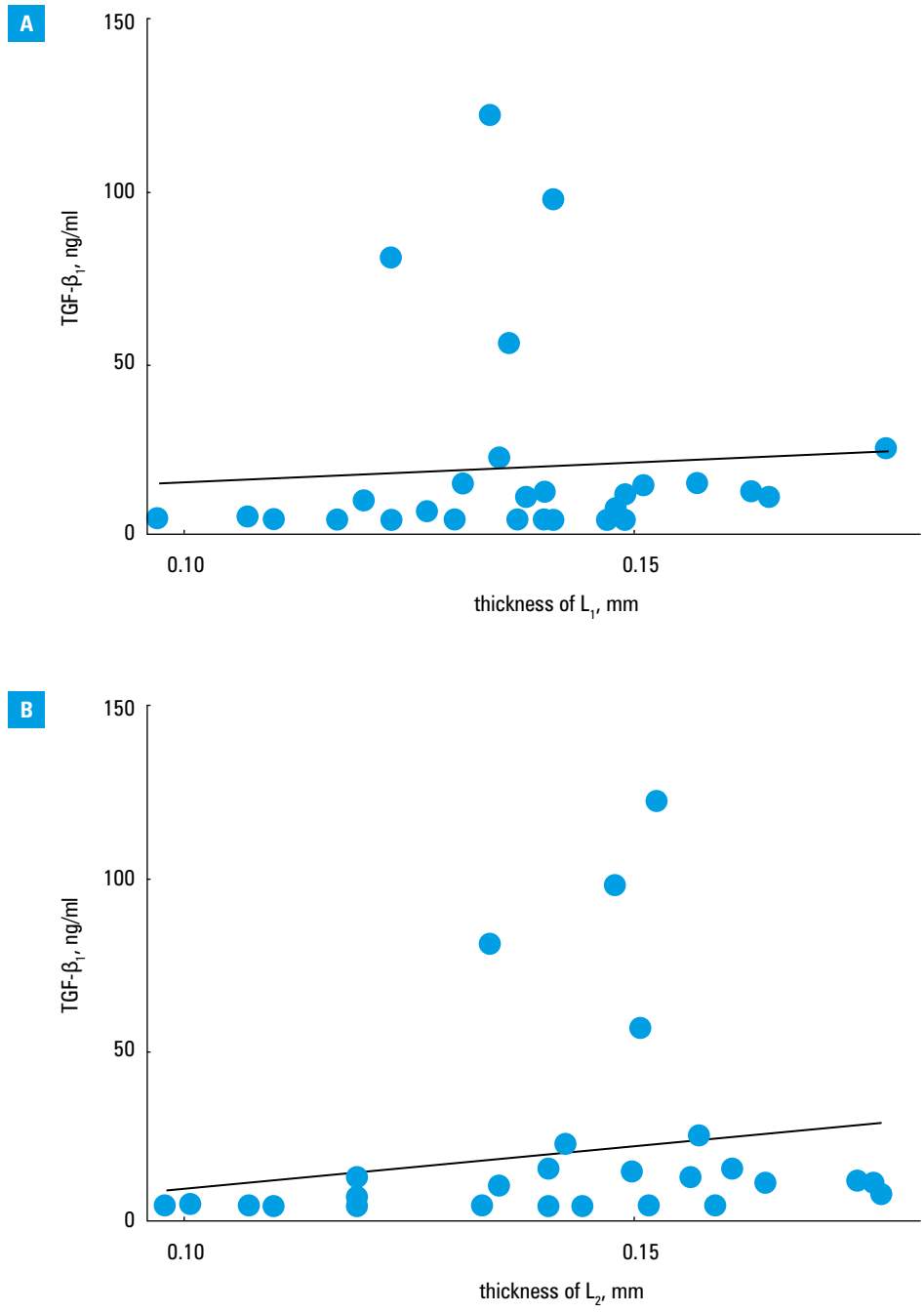


FIGURE 5 Correlation between the thickness of layers 1 (L_1; **A**) and 2 (L_2; **B**) of the bronchial wall and transforming growth factor β_1 (TGF-β_1) levels in bronchoalveolar lavage fluid

with very severe COPD and was negatively correlated with FEV_1. This is in line with previous reports showing the association between higher local[12,27] and systemic[28] levels of MMP-9 and the decrease in FEV_1. In our study, there was no association between the levels of MMP-9 or TIMP-1 in BALF, the thickness of the bronchial wall layers, and the emphysema score.

Our results are consistent with those reported by Ostridge et al,[12] in which there was no association between bronchial wall thickness and emphysema measured in CT and MMP-9. However, in a study by Boschetto et al,[1] higher concentrations of MMP-9 and MMP-9/TIMP-1 in induced sputum were observed in COPD patients with emphysema on high-resolution CT when compared with patients without emphysema, which may be due to the differences between sampling sputum or BALF.

NE is a serine protease released from the azurophil granules in response to inflammation. It is responsible for the development of emphysema by degradation of extracellular matrix components including elastin, collagens I–IV, laminin, fibronectin, and proteoglycans. By taking part in the release and activation of TGF-β_1, NE affects the development of lung tissue fibrosis.[29,30] In our study, there was a significant negative correlation between the thickness of the L_1 and NE concentrations in BALF. This may suggest that NE is responsible not only for the damage of lung tissue, but also for destruction of some connective tissue components of the bronchial wall. We did not find any association between the concentration of NE in BALF and the emphysema score. Unlike Bizeto et al,[31] who showed a negative correlation between the elevated NE level in sputum and FEV_1, we did not find any association between

NE concentrations in BALF and the results of pulmonary function tests.[31]

Study limitations Our study has several limitations. Firstly, some patients used systemic corticosteroids, which may have affected the concentrations of the studied markers. Secondly, bronchial wall thickness was measured in the third generation of the bronchi (RB10), which corresponds with large airways, while the markers of remodeling and inflammation were assessed in BALF, which mainly reflects peripheral airways. It was previously demonstrated by Nakano et al[32] that bronchial wall thickening observed on CT closely correlates with the dimensions of small airways in histological specimens and thus may indirectly indicate small airway disease. Bronchoscopy is an invasive procedure, so the studied group was relatively small, and this was associated with limited statistical power. Moreover, our group was heterogeneous in terms of dominant chronic bronchitis or emphysema and severity of the bronchial obstruction (from mild to very severe).

In conclusion, the severity of the bronchial obstruction in studied patients with COPD did not correlate either with bronchial wall thickness or with the emphysema score. It may result from the coexistence of both processes, and in some patients the inflammation of the bronchi might be dominant, while in others emphysema may be more pronounced. The significant correlation of TGF-β_1 and NE with the thickness of bronchial wall layers and the relationship of MMP-9 with the severity of bronchial obstruction may suggest contribution of the above markers to airway remodeling in COPD. We hope that further analysis of representative groups of patients with COPD would provide more reliable results and a better understanding of the mechanism of the disease.

Contribution statement JS and KS conceived the idea for the study and contributed to the design of the research project. KG, IG-S, AA, and PJ were involved in data acquisition. JS and KS performed bronchoscopy with EBUS. SM measured the total bronchial wall thickness and its layers from the images obtained during bronchoscopy. PŁ evaluated the emphysema score in chest CT scans. BJ and HP measured concentrations of the markers in BALF. AĆ analyzed the data. All authors edited and approved the final version of the manuscript.

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ARTYKUŁ ORYGINALNY

Zależność między grubością warstw ściany oskrzeli, wskaźnikiem rozedmy i markerami przebudowy dróg oddechowych w popłuczynach oskrzelowo-pęcherzykowych u chorych na przewlekłą obturacyjną chorobę płuc

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SŁOWA KLUCZOWE

markery przebudowy ściany oskrzeli, przewlekła obturacyjna choroba płuc, tomografia komputerowa, wskaźnik rozedmy, wewnątrzoskrzelowa ultrasonografia

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STRESZCZENIE

WPROWADZENIE Przebudowa ściany oskrzeli (remodeling) odgrywa ważną rolę w rozwoju przewlekłej obturacyjnej choroby płuc (POChP). Metody obrazowe, takie jak tomografia komputerowa (TK) klatki piersiowej i wewnątrzoskrzelowa ultrasonografia (*endobronchial ultrasound* – EBUS) mogą być przydatne do oceny zmian strukturalnych w płucach.

CELE Celem badania była ocena zależności między ciężkością rozedmy w TK klatki piersiowej, grubością warstw ściany oskrzeli w EBUS i markerami remodelingu w popłuczynach oskrzelowo-pęcherzykowych (*bronchoalveolar lavage fluid* – BALF) u chorych na POChP.

PACJENCI I METODY Do badania zostało włączonych 33 chorych na POChP, u których wykonano badania czynności płuc, TK z oceną wskaźnika rozedmy oraz bronchoskopię z EBUS celem pomiaru całkowitej grubości ściany oskrzeli i jej poszczególnych warstw (L_1, L_2 i $L_{3–5}$). Wybrane markery remodelingu (metaloproteinaza macierzy 9 [MMP-9], tkankowy inhibitor metaloproteinazy 1 [TIMP-1], transformujący czynnik wzrostu β_1 [*transforming growth factor* – TGF-β_1]) i zapalenia (elastaza neutrofilowa, eozynofilowe białko kationowe) oznaczano w próbkach BALF za pomocą testu immunoenzymatycznego.

WYNIKI Stężenie MMP-9 w BALF było znamiennie wyższe u chorych z bardzo ciężką obturacją niż u chorych z umiarkowaną i łagodną obturacją oskrzeli ($p = 0.02$) oraz ujemnie korelowało z wartościami natężonej objętości wydechowej pierwszosekundowej ($r = –0.538$, $p = 0.002$). Grubości warstw L_1 i L_2, histologicznie odpowiadające błonie śluzowej, podśluzowej i mięśniowej, dodatnio korelowały ze stężeniem TGF-β_1 w BALF ($r = 0.366$, $p = 0.046$ i $r = 0.425$, $p = 0.02$), a grubość warstwy L_1 wykazywała ujemną zależność z poziomem elastazy neutrofilowej ($r = –0.508$, $p = 0.004$). Nie stwierdzono korelacji między badanymi markerami w BALF a wskaźnikiem rozedmy.

WNIOSKI Istotny związek między stężeniami TGF-β_1 i elastazy a grubością warstw ściany oskrzeli oraz między stężeniem MMP-9 a ciężkością obturacji może sugerować udział tych markerów w przebudowie oskrzeli u chorych na POChP.