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Morphological Characteristics of Chronic Obstructive Pulmonary Disease (COPD) Affected Lung Tissue

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ABBREVIATIONS

CD	– cluster of differentiation
COPD	– chronic obstructive pulmonary disease
DAB	– 3'-Diaminobenzidine
ECM	– extracellular matrix
EDTA	– ethylenediaminetetraacetic acid
FEV_1	– forced expiratory volume in one second
FVC	– forced volume vital capacity
hBD	– human beta defensin
Hsp	– heat shock protein
IAA	– Institute of Anatomy and Anthropology
IgE	– Immunoglobulin E
IL	– interleukin
μm	– micrometer
MMP (MMPs)	– matrix metalloproteinase(-es)
NK	– natural killer cells
p	– p value
p/y	– pack years
WHO	– World Health Organisation
r_s	– Spearman's rank correlation coefficient (Spearman's rho)
RSU	– Rīga Stradiņš University
TGF	– transforming growth factor
Th	– T helper cell
TIMP (TIMPs)	– tissue inhibitor of metalloproteinase(-es)
TLR (TLRs)	– Toll like receptor(-s)
TNF-α	– tumour necrosis factor alpha
TRIS	– tris(hydroxymethyl)aminomethane
UK	– United Kingdom
USA	– United States of America
°	– degree
°C	– Celsius degree

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterised by progressive narrowing of airways, development of chronic inflammation, and clinically observable shortness of breath (Cazzola et al., 2007). According to data published by the World Health Organisation (WHO), COPD is the third leading cause of death in the world, accounting for about 5 % of all deaths. The prevalence of COPD worldwide is approximately 251 million cases (WHO, 2018, 2019a). Classically, COPD is associated with smoking, occupational dust, chemicals, pesticides, vapors, or other volatile contaminants (Gibson et al., 2013). Some studies have found that up to 70 % of COPD patients are non-smokers (Salvi and Barnes, 2009). In general, COPD development is associated with exposure to complex volatile gases and their particles, internal factors of an individual (heredity, local tissue changes, etc.), persistent airway irritation, poor lung development and maturation history, gender, socioeconomic status, respiratory diseases (Lange et al., 2015).

Under the influence of various risk factors, oxidative stress and chronic inflammation develop, which determines the formation and course of practically all other processes (for example, local fibrosis). Initially, combination of various factors leads to the predominance of destructive processes over tissue defense mechanisms that lead to manifestation of lung damage. Even with maximal elimination of risk factors, inflammation in COPD persists and even continues (Di Stefano et al., 2002; Bagdonas et al., 2015). Epithelial irritation causes early release of signaling molecules from epithelial cells, which further recruit other cells for inflammation. One of the most important causes and maintainers of chronic inflammation is cells with immune functions, such as neutrophils and macrophages, which receive a number of inducing factors, form and secrete proteolytic enzymes and degrade tissue components, and enhance the inflammatory response. Accordingly, the processes of chronic inflammation,

tissue destruction, and remodelling in COPD are determined by molecular and cellular mechanisms (Kobayashi and DeLeo, 2009). Even molecules formed in tissue damage initiate and sustain inflammatory processes (Salvi and Barnes, 2009; Bagdonas et al., 2015). Complex intercellular signalling pathways involving numerous cytokines and hemokines play an important role in the development of COPD, as well as remodelling processes, which in turn cause a cascade of tissue mechanisms important in pathogenesis of COPD. Further tissue changes lead to airway obstruction. The named molecular and tissue mechanisms maintain the clinical picture and symptoms characteristic of COPD, which deteriorate over time as COPD progresses (Sarir et al., 2008).

Oxidative stress or direct cell damage due to environmental factors triggers the development of an inflammatory response, which further forms the overall clinical picture of COPD. Thus, heat shock protein-70 (Hsp-70), by inducing the release of the inflammatory cytokines interleukin (IL) IL-6 and IL-8, is co-responsible for cell damage in addition to the ongoing inflammatory response (Hulina-Tomašković et al., 2019). Among the biological molecules important in the pathogenesis of COPD and their signaling, the cytokines IL-1 (IL-1α, IL-1β) and IL-33 (Gabryelska et al., 2019), IL-6, IL-8 (Wu et al., 2014, Lee et al., 2018), IL-4, IL-7 (Perotin et al., 2014; Shibata et al., 2018; Wei and Sheng Li, 2018), IL-10 (Castellucci et al., 2015), IL-12 (Zhou et al., 2018), and tumour necrosis factor-alpha (TNF-α) must be mentioned (Bai et al., 2019). The above factors determine the development of an inflammatory response that is chronic and progressive in COPD (Cho et al., 2019). Equally important are tissue remodelling events, having one of the main drivers transforming growth factor-beta (TGF-β). Hereby, TGF-β influences morphological changes in tissues, regulates the functions of the immune system and the course of inflammatory reactions, as well as participates in tissue remodelling, fibrosis, and wound healing processes. This multifunctionality is variable and changeable, primarily

in the environment of other factors (growth, inflammation, anti-inflammation, molecules modulating the inflammatory response).

The overall context of tissue changes must be taken into account when assessing TGF-β effects (Saito et al., 2018). In the case of tissue damage caused by oxidative stress and inflammatory response, extracellular matrix degradation processes are important in remodelling processes. Peptidases of tissue structural components (matrix metalloproteinases as matrix metalloproteinase-2 (MMP-2)) degrade extracellular matrix collagen, elastin, basement membrane, laminin, fibronectin (Turino, 2007). Matrix metalloproteinase tissue inhibitor-2 (TIMP-2), with its potential to promote tissue healing, is an important antagonist of MMP-2. Increased levels of TIMP-2 and TGF-β1 are associated with better tissue healing, which in turn is associated with better airway function in COPD (Ghanei et al., 2010; Gosselink et al., 2010). Antimicrobial peptides such as human beta defensins (hBDs) are an essential part of innate immunity. Human beta defensin-2 (hBD-2) has been found in various epithelia of human organs. hBD-2 interacts with other antimicrobial proteins such as lysozyme and lactoferrin (Jarczak et al., 2013), and is able to activate monocytes, macrophages, neutrophils, immature dendritic cells by hematotaxis (Winter and Wenghoefer, 2012). hBD-3 has strong antibacterial and antiviral activity, and it also acts as a hematotaxis factor (Harder et al., 2001). hBD-4 is highly antimicrobial in nature, and it is functionally more active than hBD-2 and hBD-3 (Yanagi et al., 2005).

Diagnosis of COPD and the severity of the disease are determined according to generally accepted defined diagnostic criteria. The diagnosis of COPD is based on the patient's complaints (persistent symptoms such as shortness of breath, cough with/without sputum, wheezing), assessment of the severity of the patient's complaints, objective examination (airflow disorders, including spirometry data), and risk factors (including smoking, outdoor and indoor air pollution, occupational risks). The diagnosis of COPD is also

supported by pathological changes in the airways and/or alveoli (Rabe et al., 2007; Vogelmeier et al., 2017).

The findings of bronchoscopy in COPD are variable and include local changes of chronic endobronchitis with various types of secretions (catarrhal, purulent, mucous), thickening of the bronchial wall, or, conversely, wall atrophy, and often heterogeneous findings of all changes in different bronchi (Fathy et al., 2016). Microscopic findings in COPD and bronchial asthma may be similar, including bronchial epithelial desquamation, epithelial metaplasia, variable and/or regional basement membrane thickening, mucosal and submucosal inflammation, cell infiltration (predominant with neutrophil leukocytes, CD8+ T lymphocytes and macrophage infiltration in COPD, whereas with eosinophil leukocytes in bronchial asthma), as well as bronchial gland and smooth muscle hyperplasia (Pesci et al., 1998; Arafah et al., 2018). Subjective history and lung function parameters (including severity of COPD) in COPD patients may differ from endoscopic findings and local tissue changes (Köktürk et al., 2003). Inconsistency and inaccuracy of clinical criteria for objectively observable macroscopic and microscopic tissue changes require detection of local morphopathogenic markers for accurate diagnosis of COPD.

Importantly, the studies of some markers show their linked complexes describing an associated picture of inflammation and other processes (fibrosis, tissue destruction, etc.) in COPD-affected lung tissue; however, mostly cytokines IL-1α, IL-8, TNF-α, and IL-10, remodelling marker MMP-2, growth factor TGF-β, and antimicrobial peptide hBD-2 are studied. In this study, a wide range of local factors was studied in a complex way (fifteen in total), including less studied factors – cytokines IL-1α, IL-4, IL-6, IL-8, TNF-α, IL-7, IL-12, and IL-10, growth factor TGF-β1, degradation enzyme MMP-2 and its inhibitor TIMP-2, cell and tissue oxidative stress damage marker Hsp-70, as well as hBDs hBD-2, hBD-3 and hBD-4. In general, the choice of these factors is based on the available literature, where the morphopathogenesis of COPD is determined by

cell and tissue damage under oxidative stress conditions, the formation of chronic inflammation with relevant tissue damage, as well as changes in local tissue immunity, including antimicrobial protection.

To date, contradictory and solitary data have been obtained from both the studies of animal COPD model and human COPD-affected cell cultures. In general, COPD processes have been studied mostly in a small number of patients in tissue fluids and cell cultures, which are relatively difficult to attribute to true tissue processes. Studies also lack an ontogenetic aspect.

The aim of the study was to determine and evaluate the morphological characteristics of COPD-affected lung tissue by analysing the relative abundance and distribution of markers relevant to chronic inflammation, tissue remodelling changes and local protective mechanisms, including ontogenetic perspective.

In order to conduct the research, the following **study objectives** were set:

1. To evaluate the morphological overview of bronchial and alveolar structures in lung tissue of COPD patients by routine histological staining method.
2. To determine the appearance and relative distribution of inflammatory cytokines IL-1α, IL-4, IL-6, IL-8, TNF-α, regulatory cytokines IL-7 and IL-12, anti-inflammatory cytokine IL-10, growth factor TGF-β1, degradation enzyme MMP-2 and its inhibitor TIMP-2, cell and tissue damage marker Hsp-70, also antimicrobial protection factors hBD-2, hBD-3 and hBD-4 in the relatively healthy control group lung tissue by immunohistochemistry (IHC).
3. To determine the appearance and relative distribution of inflammatory, regulatory and anti-inflammatory cytokines IL-1α, IL-4, IL-6, IL-8, TNF-α, IL-7, IL-12, IL-10 in COPD-affected lung tissue by IHC.
4. To determine the appearance and relative distribution of growth factor TGF-β1, degradation enzyme MMP-2 and its inhibitor TIMP-2, cell and tissue damage marker Hsp-70 in COPD-affected lung tissue by IHC.
5. To determine the appearance and relative distribution of antimicrobial factors hBD-2, hBD-3 and hBD-4 in COPD-affected lung tissue by IHC.

6. To perform processing of the data and statistical analysis; to compare the acquired morphological data between COPD-affected lung tissue and the relatively healthy control group lung tissue.
7. To evaluate the relationship of morphological changes of COPD-affected lung tissue with the clinical findings of COPD patients by correlating the acquired morphological data with medical history data, functional indicators, as well as bronchoscopic findings.
8. To determine the ageing changes in lung tissue of the control group and COPD patients by correlating the IHC findings of different tissue factors with age and in different age groups.

Hypotheses of the study. COPD morphopathogenesis is affected by changes in the relative abundance and distribution of chronic inflammation, tissue remodelling, cell and tissue damage-associated factors, and local tissue protection markers, as shown by the interaction of these markers in the complex relationship. The relative abundance and distribution of various tissue factors in the lung tissue of healthy people and COPD patients changes with age.

Novelty of the study. This study describes the detection of cytokines, remodelling factors, and antimicrobial protection factors in the lung of relatively healthy patients and COPD affected patients using IHC. In general, to date, complex data on the findings of morphology and immunoreactive structures in relatively healthy and COPD-affected bronchial tissues, indicating local changes in the tissues, have been studied poorly in scientific publications. Markers such as interleukins and others have been studied as extensively in most publications. are considered alone or in combinations of some markers. For example, the course of chronic inflammation locally in tissues also affects remodelling processes, as indicated by the interaction of these factors. A number of factors have been identified, the location and interaction of which indicate much broader

functions than described so far. The presence of various factors in terms of ageing in the lung tissues of relatively healthy individuals and COPD patients, which is an important part of this study, has been little studied. In the case of age-related general inflammation, there are limited data on lung ageing and local markers associated with the inflammatory response. In recent years, there has been a small number of studies conducted in Latvia in the field of pulmonology or on the health of the respiratory system. Thus, the current study on the discovery of complex factors in the lung tissues of COPD patients is considered to be an innovative and practically extensive one carried out so far. The above mentioned statements determine the topicality of the research and substantiates the significance of the research in morphology, fundamental and clinical pulmonology.

Individual contribution. The Author participated in all stages of the study, performed the planning of research work, routine histological and IHC reactions, analysis of the tissue material and the acquisition of scientific data, performed the statistical analysis of all data. The Author has written all this work and is the author of all the microphotographs.

Ethical aspects. All authors hereby declare that all study performances were examined and appropriately approved by the by the local Ethical Committee of Pauls Stradins Clinical University Hospital, and were therefore implemented in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki (Ethical code number: 230113-17L, approval date: January 23, 2013).

Structure and volume of the Doctoral Thesis. The Doctoral Thesis is written in Latvian. It consists of 5 chapters: literature review, material and methods, results, discussion and conclusions. The volume of the Doctoral Thesis is 290 pages, the work contains 42 tables, 65 graphs and 60 figures (microphotographs). The references of the Doctoral Thesis consists of 419 sources.

1 MATERIALS AND METHODS

1.1 Tissue Material Used for Morphological Analysis

The study population consisted of 40 patients with a clinically and functionally established diagnosis of COPD (mild to severe COPD). The diagnosis of COPD and the severity of the disease were determined according to generally accepted defined diagnostic criteria. Of all COPD patients, lung tissue material was obtained during fibrobronchoscopy under local anesthesia with videobronchoscope from 36 patients, obtaining several bronchial mucosa biopsies from the right lobe segmental bronchi for diagnostic purposes (2012–2018). Tissue material from remaining four patients was obtained from the historical tissue material collection of the Institute of Anatomy and Anthropology (IAA), Rīga Stradiņš University (RSU) (1998–1999).

Of the study COPD patients, 39 were male and one was female. All COPD patients ranged in age from 53 to 88 years. The duration of COPD (in years) in 34 patients ranged from 1 year to 25 years. The fact of smoking was calculated in 35 COPD patients with a calculated number of pack years (p/y), which ranged from 10 p/y to 70 p/y. Lung functional measurements of FEV_1 (forced expiratory volume per second; percentage (%) and units of volume (litres)), FVC (forced vital capacity; percentage (%) and units of volume (litres)) were determined by spirometry in 35 patients, also the Tiffno index (Tiffeneau-Pinelli index) or the ratio between FEV_1 and FVC. COPD patients overall had FEV_1 (%) from 20.4 to 100, FEV_1 (L) from 0.61 to 3.10, FVC (%) from 19.33 to 95.8, FVC (L) from 0.74 to 4.55, FEV_1/FVC from 0.289 to 0.7251.

Of all COPD patients, 36 patients had hypertrophic or atrophic changes in the bronchial wall, chronic endobronchitis, as well as airway deformities at the bronchoscopic site.

Control group lung material was obtained in a post-mortem autopsy from 49 individuals of various ages who died of accidents and not from respiratory

disease, or during lung surgery. The control group included 37 men and 12 women aged nine to 95 years. The tissue material was obtained from the historical tissue material collection of the IAA, RSU (1993–1995). At the autopsy, bronchial wall tissue material was obtained from the large cartilagineous bronchi (main and lobar), which also contained lung parenchyma material. Among the diagnoses of the control subjects, mostly sudden cardiac death (sudden cardiac arrest), intentional selfharm (suicide) and unintentional major injury due to trauma (vehicle crash, traffic collision) were dated. Control group tissue samples with medical data records and histopathological findings conflicting the possible results were excluded.

1.2 Methods of Morphological Analysis

1.2.1 Tissue Material Fixation and Section Preparation

For routine histological and immunohistochemical methods, the tissue material was processed in the Laboratory of Morphology, IAA, RSU.

Approximately 1 cm^3-sized specimens of lung tissue were obtained. Tissue material was fixed in Stefanini (Zamboni) tissue fixation solution (Stefanini et al., 1967). Storage was performed in the refrigerator. After fixation, the tissue material was delivered to the Laboratory of Morphology, where it was rinsed with Tyrode's solution for 24 hours, and increasing concentration alchohol solutions (50º–95º) were used for dehydration and degreasing for 30 minutes, 1–2 hours, 3–4 hours, 24 hours. The tissue material was purified using two continuous washes of the xylene solution for 1 hour. The fixed tissue material was prepared for embedding in paraffin blocks. The paraffin mixture was homogenised and the tissues were infiltrated for 3 hours by applying two paraffin sets. The tissue material embedded in the molten paraffin was solidified in a paraffin block, from which tissue sections were further prepared for routine histology staining and immunohistochemistry. The tissue material in the paraffin

block was cut into serial 3–4 μm thick sections using a laboratory semi-automatic rotating microtome (code Leica RM2245; Leica Biosystems Richmond, USA). Sections of tissue material from the microtome razor were transferred to water (48–50 ºC) and then to a slide (code 6130603; Histobond®+, Paul Marienfeld GmbH & Co. KG, Germany). Tissue sections applied to slides were placed in a thermostat at 56 ºC for 20–60 minutes.

1.2.2 Routine Histological Staining Method

Tissue sections were prepared for routine histological staining by deparaffinisation. Paraffin from tissue micropreparations was rinsed with two continuous rinses of xylene solution for 10 minutes. Then tissue material was dehydrated using 96º ethanol solution. The prepared tissue sections were stained with hematoxylin (code 05-M06002, Mayer's; Bio Optica Milano, Italy) and eosin (kods 05-B10003; Mayer's; Bio Optica Milano, Italy). Tissue sections stained with hematoxylin and eosin for routine histological staining provided the morphological overview. In the overview sections, the basophilic structures of the cells and tissues turned bluish purple, but the acidophilic structures turned pink (Lillie et al., 1976). After staining, the tissue material was dehydrated with 70º–96º ethanol for 9 minutes, clarified with carboxylol and xylene. A drop of histological glue (Pertex glue (code Lot 1710013, Histolab Products AB, Sweden)) was applied to the stained tissue sections and covered with a 0.13–0.16 mm cover glass (code H875.2; Carl Roth GmbH+Co, Germany). Tissue material for routine histological staining was analysed by bright field light microscopy with a Leica DC 300F camera microscope (kods Leica DM500RB, Leica Biosystems Richmond, USA).

The morphological evaluation of the tissue material prepared by the routine histologal staining method was performed by analysing the following findings: bronchial pseudostratified epithelium (epithelial cell desquamation,

metaplasia, epithelial cell hyperplasia, inflammatory cell infiltration); basement membrane (thickened basement membrane); mucosal connective tissue (fibrosis, granulation tissue, inflammatory cell infiltration assessed in the bronchial wall and/or perivascularly according to the following scheme: 1) no infiltration of inflammatory cells is observed (score 0); 2) minimal, local infiltration of individual cells (grade 1); 3) moderate inflammatory cell infiltration (score 2); 4) significant infiltration of inflammatory cells (grade 3); bronchial glands (hypertrophy, inflammatory cell infiltration); bronchial smooth muscle (hyperplasia); microcirculatory blood vessels (thickened vascular wall, perivascular fibrosis).

1.2.3 Immunohistochemistry Method and Reagents

Appearance and relative distribution of inflammatory, regulatory and anti-inflammatory cytokines, tissue remodelling and antimicrobial protection factors were determined in tissue sections of relatively healthy control and COPD patients using the biotin-streptavidin immunohistochemistry method (Hsu et al., 1981; Mori and Cardiff, 2016). Data on the antibodies used in the study have been summarised in Table 1.1.

Table 1.1

Antibodies used in immunohistochemistry

No.	Antibody	Code	Obtained from	Working dilution	Manufacturer
1.	IL-1α	sc-9983	Mouse	1:50	Santa Cruz Biotechnology, USA
2.	IL-4	orb10908	Rabbit	1:100	Biorbyt Ltd., UK
3.	IL-6	sc-73319	Mouse	1:50	Santa Cruz Biotechnology, USA
4.	IL-7	orb48420	Rabbit	1:100	Biorbyt Ltd., UK
5.	IL-8	sc-1269	Goat	1:50	Santa Cruz Biotechnology, USA
6.	IL-10	P22301	Rabbit	1:400	Nordic BioSite, Sweden

Continuation of the table

7.	IL-12	orb10894	Rabbit	1:100	Biorbyt Ltd., UK
8.	TNF-α	ab6671	Rabbit	1:100	Abcam, UK
9.	TGF-β1	orb7087	Rabbit	1:100	Biorbyt Ltd., UK
10.	MMP-2	sc-53630	Mouse	1:100	Santa Cruz Biotechnology, USA
11.	TIMP-2	sc-21735	Mouse	1:100	Santa Cruz Biotechnology, USA
12.	Hsp-70	585054A	Mouse	1:100	Invitrogen, Italy
13.	hBD-2	sc-20798	Rabbit	1:200	Santa Cruz Biotechnology, USA
14.	hBD-3	rb183268	Rabbit	1:100	Biorbyt Ltd., UK
15.	hBD-4	ab14419	Mouse	1:200	Abcam, UK

Abbreviations in the table: IL-1α, -4, -6, -7, -8, -10, -12 – interleukin-1α, -4, -6, -7, -8, -10, -12; TNF-α – tumour necrosis factor-alpha; TGF-β1 – transforming growth factor-beta 1; MMP-2 – matrix metalloproteinase-2; TIMP-2 – MMP-2 tissue inhibitor; Hsp-70 – heat shock protein-70; hBD-2, -3, -4 – human beta defensin-2, -3, -4.

Tissue sample fixation and preparation, embedment into paraffin blocks, microtomy and placement on slides was performed according to the scheme described above (see Section 1.2.1). Then 3–4 μm thick tissue sections were obtained from the paraffin block of the tissue material, transferred to a slide, and dried in a thermostat. Tissue sections were then deparaffinised with xylene solution and dehydrated with graduated alcohol solutions. Deparaffinised, washed, and cleared tissue sections were placed in a holder and rinsed with TRIS buffer (code 2017X12508; Diapath, Italy) for 10 minutes. Tissue sample was boiled in EDTA (pH 9,0) buffer (code 2017X02239; Diapath, Italy) in the microwave for 10 minutes. Afterwards, the container with the tissue samples was cooled to 65 °C. A holder with tissue samples was placed in TRIS wash buffer. Endogenous peroxidase was blocked with a 3 % peroxidase block (code K400611, Dako, Denmark) for 10 minutes. Rinsing with TRIS wash buffer was performed for 10 minutes. All antibodies used in the study were diluted with Antibody Diluent (code 938B-05; Cell MarqueTM, USA).

The HiDef Detection™ HRP polymer system was used for mouse or rabbit antibodies (code 954D-30, Cell MarqueTM, USA). Incubation with primary antibody was performed for 2 hours. Tissue samples were washed in TRIS wash buffer for 10 minutes. The HiDef Detection™ reaction amplifier was then used (code 954D-31; Cell MarqueTM, USA) at room temperature for 10 minutes. The tissue material was then rinsed in TRIS wash buffer for 10 minutes. The HiDef Detection™ HRP polymer label (code 954D-32; Cell MarqueTM, USA) was then used at room temperature for 10 minutes.

The ImmunoCruz™ ABC staining system was used for goat antibodies (code sc-2023; Santa Cruz Biotechnology, USA). It includes biotin-containing secondary and tertiary antibodies. Tissue material was incubated in 1.5 % blocking serum in TRIS buffer for up to one hour at room temperature. Tissue material was incubated with the primary antibody for up to one hour. Tissue material was rinsed in TRIS wash buffer for 10 minutes. Tissue sections were incubated with biotin-containing secondary antibody for 30 minutes and rinsed again for 10 minutes in TRIS wash buffer. Tissue sections were incubated with biotin-containing tertiary antibody for 30 minutes.

All immunohistochemically treated tissue sections were rinsed in TRIS wash buffer for 10 minutes. The tissue sections were coated with the DAB+ chromogenic liquid DAB Substrate Kit (code 957D-60; Cell MarqueTM, USA), as well as incubated at room temperature for up to 10 minutes to obtain a brown staining of immunoreactive structures. The sections were then washed in distilled water for 5 minutes. Tissues were then contrast stained with hematoxylin (code 05-M06002, Mayer's Bio Optica Milano, Italy) for 2 minutes. Further, the antibody-treated tissue material was dehydrated with 70º–96º ethanol solutions. Tissue sections were clarified with carboxylol and xylene. A drop of Pertex glue was applied to the tissue section and a coverslip was glued.

Negative and positive control sections were prepared for this study.

1.3 Data Processing Methods

Tissue material for routine histological staining was analysed by bright field light microscopy with a Leica DC 300F camera microscope. The obtained images were analysed with the image processing software Image Pro Plus 6.0 (Media Cybernetics, USA). Image processing programmes Photo Pos Pro 3.2 (Power Of Software LTD., Israel) and paint.net (dotPDN LLC and Rick Brewster, USA) were used for image visualisation and processing.

1.3.1 Semiquantitative Counting Method

Quantification of immunoreactive structures (cells) was performed using a semi-quantitative counting method (Tobin et al., 1990; Pilmane et al., 1998):

0 – no immunoreactive structures were detected in the visual field;

0/+ – occasional positive structures in the visual field;

+ – few positive structures in the visual field;

+/++ – few to moderate positive structures in the visual field;

++ – moderate positive structures in the visual field;

++/+++ – moderate to numerous positive structures in the visual field;

+++ – numerous positive structures in the visual field;

+++/++++ – numerous to abundant positive structures in the visual field;

++++ – abundance of positive structures in the visual field.

Cells whose cell nucleus/cytoplasm was marked brown in an IHC were considered immunoreactive (immunopositive). Immunoreactive structures (cells) were analysed in the following tissue groups and localisations of lung material: bronchial epithelium; bronchial mucosal connective tissue; wall of mucosal microcirculation blood vessels; bronchial smooth muscle; bronchial glands; bronchial hyaline cartilage; alveolar epithelium; alveolar macrophages. Immunoreactive (positive) structures were evaluated in five randomly selected fields of view at 400 X magnification for each tissue section material.

1.3.2 Data Statistical Analysis

Statistical processing was performed by creating a ranking (ordinal value) scale and categorising the obtained data according to the number of immunoreactive cells counted from 0 (no immunoreactive cells were detected) to 4.0 (abundance of immunoreactive cells). The normal distribution of the data was tested by the Kolmogorov-Smirnov normality test. Non-parametric statistical methods were used for statistical analysis of immunohistochemical data. The median and interquartile amplitudes were evaluated. The Wilcoxon matched pairs Signed Rank Test was used to compare two dependent groups. The Mann-Whitney U Test was used to determine the differences between the number of immunoreactive cells of each examined marker within different lung tissue compartments and localisations in COPD patients compared to control patients. Spearman's Rank Order Correlation was used in the analysis of the correlation between the two parameter groups. The classification of the correlation strength according to the value of the correlation coefficient r_s was as follows: $|r_s|$ = 0–0.3 – insignificant, $|r_s|$ = 0.3–0.5 – weak, $|r_s|$ = 0.5–0.7 – moderate, $|r_s|$ = 0.7–0.9 – moderately strong, $|r_s|$ = 0.9–1.0 – strong correlation. In all data statistical processing methods, the results were considered statistically significant if the p value is < 0.05. Statistical processing and visualisation was performed with IBM SPSS statistical software 23rd version (IBM Company, USA) and GraphPad Prism 8.3.0 software (GraphPad Software, USA).

1.4 Definition of age groups in analysis of age changes

The age changes analysed in this study were determined by age groups 9–20 years, 21–34 years, 35–49 years, 50–64 years, 65–74 years, ≥ 75 years (Halbert et al., 2006; Moliva et al., 2014; Thannickal et al., 2015; Budinger et al., 2017; WHO, 2019b).

2 RESULTS

2.1 Characterisation of morphological findings in control patients and patients with chronic obstructive pulmonary disease

All 49 control patients had a large bronchus wall in their sections, and 25 control patients also had additional lung parenchyma (alveolar structures) in their sections. In the lung tissue material of all 49 control group patients, a practically unchanged lung histological picture in accordance with the generally accepted relative norm was found.

Variable and different grades and localisations of chronic inflammation and tissue remodelling were visualised in all lung tissue materials of 40 COPD patients. The morphological finding of COPD patient's tissue material included infiltration of inflammatory cells into the bronchial epithelium, mucosal connective tissue and bronchial glands, also granulation tissue, thickened basement membrane, bronchial gland and smooth muscle hypeplasia and hypertrophy, and thickened walls of blood vessels. Changes in remodelling were indicated by fibrosis, the presence of pronounced and thick bundles of connective tissue fibres, as well as an overall thickened bronchial wall.

Different morphological findings of bronchial epithelium in the tissue material of all 40 COPD patients were visualised. Fragmented desquamation of epithelial cells were found in the tissue material of all patients. Of all 40 COPD patients, 24 patients had bronchial epithelium fragments of normal epithelium, and none of the altered epithelium findings was found in the tissue material of one patient; basal epithelial cell hyperplasia was fragmentarily detected in the bronchial epithelium of 37 patients; thick mucoid epithelium was visualised in 19 patients; epithelial metaplasia was observed in 23 patients. Interestingly, different findings of epithelial changes were combined in every patient's tissue material. In all COPD patients, the finding of **epithelial metaplasia** was associated with worse and/or more variable functional parameters FEV_1 (L)

(U = 50, p < 0.001), FEV_1 (%) (U = 75, p = 0.009), FVC (L) (U = 64.5, p = 0.003), FEV_1/FVC (U = 69, p = 0.009) and with COPD severity (U = 96, p = 0.037).

In the mucosal connective tissues of 34 COPD patients, fibrotic changes with large, densely packed connective tissue collagen fiber bundles were found, between which rare connective tissue cells were visualised. In some patients, bundles of collagen fibers were arranged chaotically and irregularly. In COPD patients, the presence of **fibrosis** in the histological sections of the report was associated with poorer functional parameters (FEV_1 (L) (U = 36.5, p = 0.024), FEV_1 (%) (U = 41, p = 0.044), FVC (L)) (L) (U = 40.5, p = 0.04).

Inflammatory cell infiltration was observed in the bronchial epithelium and connective tissue of all 40 COPD patients. Widespread inflammatory infiltrates containing lymphocytes, neutrophils, and macrophages ranged from minimal, local (1) to significant (3) infiltration of inflammatory cells in the bronchial tissue of all COPD patients. Of all COPD patients, 14 had bronchial tissue infiltrates of the eosinophils in the bronchial epithelium and/or connective tissue. Infiltration of lymphocytes and neutrophils was detected in the bronchial epithelium. In general, infiltration of inflammatory cells into the bronchial epithelium was not pronounced (1). Infiltrates of neutrophils, lymphocytes, macrophages, eosinophils and plasma cells were found in the connective tissue of the bronchial mucosa. In general, inflammation cell infiltration in connective tissue was moderate (2), while in the glands it was minimal (1). The **degree of infiltration of inflammatory cells in connective tissue** was statistically significantly more pronounced than in epithelium (Z = –3.448, p = 0.001) and glands (Z = –4.433, p < 0.0001).

In addition to inflammatory cell infiltrates in mucosal connective tissue, granulation tissue was also visualised in 28 COPD patients. **Granulation tissue** finding was associated with more pronounced infiltration of inflammatory cells

into connective tissue (U = 95.5, p = 0.031). Moreover, granulation tissue finding was associated with a longer smoking history (p/y) (U = 60, p = 0.017).

2.2 Characterisation and statistical analysis of biotin-streptavidin immunohistochemistry data

2.2.1 Immunohistochemistry data and statistical analysis of various tissue factors in control group patients

Immunohistochemistry data of various tissue factors in control patients

Inflammatory, regulatory and anti-inflammatory cytokines

Interleukin-1 alpha (IL-1α). Overall, in most (36) control patients, none (0) or occasional (0/+) IL-1α immunoreactive cells in the bronchial epithelium and connective tissue were found. In majority (36) of the control group patients, cells containing this factor were not detected in the blood vessels, muscles and glands (0), while in the cartilage of most control patients (16), few (+) IL-1α immunoreactive cells were detected. The numbers of IL-1α immunoreactive cells in the alveolar lung material of control patients varied markedly from none (0) to numerous (+++).

Interleukin-4 (IL-4). In general, few (+), few to moderate (+/++) and moderate (++), as well as numerous (+++) IL-4 immunoreactive cells were detected in the bronchial epithelium of most control patients (26). In majority of control patients, connective tissue (44), blood vessels (38), bronchial smooth muscle (33), glands (33) and cartilage (31) contained none (0) or occasional (0/+), or few (+) IL-4 immunoreactive cells. In the alveolar epithelium and among alveolar macrophages, the amount of IL-4-containing cells ranged from none (0) to numerous to abundant (+++/++++).

Interleukin-6 (IL-6). Occasional (0/+), few (+), and few to moderate (+/++) IL-6 immunoreactive cells were found in the bronchial epithelium of most (25) control patients. All (40) control patients had none (0) or occasional (0/+) IL-6-immunoreactive cells in connective tissue, blood vessels, muscle, and glands, while most of them (22) had few (+) to moderate (++) IL-6 positive cells in cartilage. Occasional (0/+) to moderate to numerous (++/+++) IL-6-containing cells in the alveolar epithelium and among alveolar macrophages were identified in part of control patients (24).

Interleukin-8 (IL-8). In majority of control patients (37), bronchial epithelium and muscle lacked (0) or there were mostly detected occasional (0/+) to few (+) IL-8 immunoreactive cells. In all control patients, the amount of IL-8 immunoreactive cells ranged from none (0) to numerous (+++) in connective tissue, blood vessels, glands, cartilage, alveolar epithelium and among alveolar macrophages.

Tumour necrosis factor-alpha (TNF-α). In most control patients, TNF-α immunoreactive cells were either not found (0) in the bronchial epithelium (37), mucosal connective tissue (41), blood vessels (43), smooth muscle (42), glands (40), and cartilage (31), or mostly occasional (0/+) and few (+) TNF-α-containing cells were identified. In part of control patients, the number of TNF-α-positive cells in the alveolar lung material ranged from none (0) to few (+) in the alveolar epithelium, as well as to numerous (+++) among alveolar macrophages.

Interleukin-7 (IL-7). Overall, in majority of control patients (28), few (+) to moderate (++) numbers of IL-7-containing cells were detected in the bronchial epithelium. In the connective tissue, blood vessels, bronchial smooth muscle and glands of most control patients (44), none (0), occasional (0/+) and few (+) IL-7

immunoreactive cells were found, whereas in cartilage (30), mostly moderate (++) to numerous (+++) of those were detected. In alveolar epithelium and among alveolar macrophages, the amount of IL-7-containing cells varied markedly from their deficiency (0) to as much as to numerous to abundance (+++/++++).

Interleukin-12 (IL-12). In majority of control patients (33), few (+), few to moderate (+/++), to moderate (++) numbers of IL-12 immunoreactive cells were detected in the bronchial epithelium. None (0) to occasional (0/+), to few (+) IL-12 immunoreactive cells were found in the connective tissue and blood vessels of majority of control patients (33). In the bronchial smooth muscle, glands and cartilage (36), mostly few (+) and few to moderate (+/++) IL-12-containing cells were detected. For most patients, occasional (0/+) to few (+) IL-12 positive cells in the alveolar epithelium and among alveolar macrophages were detected.

Interleukin-10 (IL-10). In part of control patients (22), few (+) and few to moderate (+/++) numbers of IL-10 immunoreactive cells were detected in the bronchial epithelium, but none (0) to occasional (0/+), to few (+) were found in connective tissue, blood vessels, smooth muscle and glands. In the bronchial cartilage (24), mostly few to moderate (+/++) and moderate (++) numbers of IL-10 immunoreactive cells were detected. Mostly (22) occasional (0/+), few (+), few to moderate (+/++), and moderate (++) IL-10 immunoreactive alveolocytes and alveolar macrophages were counted.

Lung tissue remodelling factors

Transforming growth factor-beta 1 (TGF-β1). Occasional (0/+) and few (+) TGF-β1 immunoreactive cells were detected in the bronchial epithelium of majority of control patients (22); however, in connective tissue (47), blood

vessels (39), smooth muscle (33) and glands (33), mostly occasional (0/+) and few (+) cells were identified. In cartilage, few (+), few to moderate (+/++), to moderate (++) TGF-β1 positive cells were mostly (35) detected. In turn, in the alveolar epithelium and among alveolar macrophages, mostly (25) occasional (0/+), few (+), few to moderate (+/++) TGF-β1-containing cells were detected.

Matrix metalloproteinase-2 (MMP-2). In most control patients, none (0), occasional (0/+), few (+) and few to moderate (+/++) numbers of MMP-2 immunoreactive cells were detected in the bronchial epithelium (23), connective tissue (47), vascular wall (34), smooth muscle (27) and glands (37). In the bronchial cartilage, a variable number of MMP-2 positive cells were found from none (0) to numerous (+++). Occasional (0/+), few (+), few to moderate (+/++), and moderate (++) numbers of MMP-2 positive cells were detected in the alveolar epithelium and among alveolar macrophages in part of control patients (21).

Tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). In bronchial epithelium (30), mucosal connective tissue (46), vascular wall (43), smooth muscle (33), and glands (39) of most control patients, none (0) of TIMP-2 immunoreactive cells or occasional (0/+), few (+) and few to moderate (+/++) numberos of factor positive cells were found. In most of control patients (35), few to moderate (+/++), moderate (++), and moderate to numerous (++/+++) TIMP-2 immunoreactive cells were detected in cartilage. In alveolar epithelium and among alveolar macrophages, mostly (21) few (+), few to moderate (+/++), and moderate (++) TIMP-2 immunoreactive cells were found.

Indicators of cellular and tissue oxidative stress

Heat shock protein-70 (Hsp-70). In part of control patients (20), mostly few (+) Hsp-70 immunoreactive cells were detected in the bronchial epithelium. Mostly

none (0), occasional (0/+) and few (+) Hsp-70-containing cells were found in connective tissue (47), blood vessels (38), smooth muscle (32) and glands (37). In most control individuals (26), few to moderate (+/++) and moderate (++) Hsp-70 immunoreactive cells were detected in cartilage. Mostly (21) few (+), few to moderate (+/++) and moderate (++) Hsp-70 positive cells were found in alveolar epithelium and among alveolar macrophages.

Local antimicrobial protection factors

Human beta defensin-2 (hBD-2). In majority of control patients (34), none (0) or occasional (0/+) hBD-2 immunoreactive cells in the bronchial epithelium, connective tissue, blood vessels, muscle, and glands were found. In most control individuals (33), few to moderate (+/++) to numerous (+++) numbers of hBD-2 immunoreactive cells were detected in cartilage. In part of control patients (21), in alveolar epithelium and among alveolar macrophages, occasional (0/+) to moderate (++) numbers of hBD-2 positive cells were detected altogether.

Human beta defensin-3 (hBD-3). In majority of control patients (28), none (0) or occasional (0/+) hBD-3 immunoreactive cells were found in the bronchial epithelium, connective tissue, blood vessels, smooth muscle, and glands. In cartilage (31), alveolar epithelium and among alveolar macrophages (18) of most control patients, occasional (0/+) to moderate (++) numbers of hBD-3 immunoreactive cells were found.

Human beta defensin-4 (hBD-4). In majority of control patients, none (0) or only occasional (0/+) hBD-4-containing cells were found in the bronchial epithelium (31), connective tissue (32), blood vessels (26), muscle (39), and glands (30). In cartilage (33), alveolar epithelium (21), and among alveolar macrophages (15) of part of control individuals, occasional (0/+) to moderate (++) numbers of hBD-4 immunoreactive cells were detected.

In general, less immunoreactive cells were determined in the connective tissue, blood vessels, smooth muscle, as well as in glands of the control group patients. Of particular note, statistically significant ($p < 0.05$) lower numbers of **IL-6** and **Hsp-70** immunoreactive cells was found in smooth muscle.

In the tissue material of the control group patients, more immunoreactive cells were determined in the bronchial epithelium, cartilage, alveolar epithelium, and among alveolar macrophages. Separately from this finding, statistically significantly ($p < 0.05$) higher numbers of **IL-4**, **IL-7** immunoreactive cells in bronchial epithelium, higher numbers of **MMP-2**, **TIMP-2**, **hBD-2**, **hBD-3**, **hDB-4** immunoreactive cells in bronchial cartilage, as well as a higher numbers of **IL-8**, **TNF-α** and **TGF-β1** immunoreactive cells among alveolar macrophages were found. The number of **IL-8** immunoreactive cells in blood vessels and the number of **TNF-α** immunoreactive cells in connective tissue were also statistically significantly ($p < 0.05$) higher, but the number of **IL-12**-containing alveolar macrophages was lower (see Table 2.1).

Evaluation of relationship between different tissue factors in control patients

Statistically significant ($p < 0.05$) correlations between the numbers of **IL-1α**, **IL-4**, **IL-6**, **TNF-α**, **IL-7**, **IL-10** immunoreactive cells in the bronchial and alveolar epithelium, as well as among alveolar macrophages should be mentioned separately. In addition, statistically significant ($p < 0.05$) correlations were also determined for the numbers of **TIMP-2**, **Hsp-70**, **TGF-β1**, **hBD-2**, **hBD-3**, **hBD-4** immunoreactive cells among alveolar macrophages.

Table 2.1

Relative appearance and distribution of various tissue factors in lung tissues of control patients

	Control group patients (N = 49)							
	E.	**C.t.**	**B.v.**	**S.m.**	**G.**	**C.**	**A.e.**	**A.m.**
IL-1α	0/+	0/+	0	0	0	+	0/+	+
IL-4	+/++ *	0/+	0/+	0	0/+	0/+	+	+/++
IL-6	+	0/+	0/+	0 *	0	+	+	+/++
IL-8	0/+	+	++ *	0/+	+	++/+++	+	++ *
TNF-α	0	0/+ *	0	0	0	0/+	0/+	+/++ *
IL-7	+/++ *	0/+	+	0	0/+	++	+	+/++
IL-12	+/++	0/+	+	+/++	+/++	+	+	0/+ *
IL-10	+/++	+	+	0/+	0/+	++	+	+/++
TGF-β1	+	0/+	0/+	0	0/+	+	+	+ *
MMP-2	+ *	0/+	0/+	0	0/+	+/++ *	+	+ – +/++
TIMP-2	+	0/+	+	0/+	0/+	++ *	+/++	+/++
Hsp-70	+	0/+	0/+	0 *	0/+	+/++	+/++	+
hBD-2	0/+	0/+	+	0	0/+	++ *	+/++	+/++
hBD-3	0/+	0/+	0/+	0/+	0/+	+/++ *	+/++	+
hBD-4	0/+	0/+	0/+	0	0	+ *	+/++	+

Abbreviations in the table: E. – bronchial epithelium; C.t. – mucosal connective tissue; B.v. – mucosal blood vessels; S.m. – smooth muscle; G. – bronchial glands; C. – bronchial cartilage; A.e. – alveolar epithelium; A.m. – alveolar macrophages; IL-1α, -4, -6, -7, -8, -10, -12 – interleukin-1α, -4, -6, -7, -8, -10, -12; TNF-α – tumour necrosis factor-α; TGF-β1 – transforming growth factor-β1; MMP-2 – matrix metalloproteinase-2; TIMP-2 – tissue inhibitor of MMP-2; Hsp-70 – heat shock protein-70; hBD-2, -3, -4 – human beta defensin-2, -3, -4; 0 (0) – no immunoreactive cells (i.c.) in visual field (v.f.); 0/+ (0.5) – occasional i.c. in v.f.; + (1.0) – few i.c. in v.f.; +/++ (1.5) – few to moderate i.c. in v.f.; ++ (2.0) – moderate i.c. in v.f.; ++/+++ (2.5) – moderate to numerous i.c. in v.f.; N – patient count; * – statistically significant difference ($p < 0.05$).

2.2.2 Immunohistochemistry data and statistical analysis of various tissue factors in COPD patients

Immunohistochemistry data of various tissue factors in control patients

Inflammatory, regulatory and anti-inflammatory cytokines

Interleukin-1 alpha (IL-1α). In the bronchial epithelium of most COPD patients (23), moderate to numerous (++/+++), to numerous (+++), to numerous to abundance (+++/++++) of IL-1α-containing cells were foundd; however, in connective tissue (18), blood vessels (19), smooth muscle (34) and glands (21) – occasional (0/+) to few (+) IL-1α immunoreactive cells (Figure 2.1).

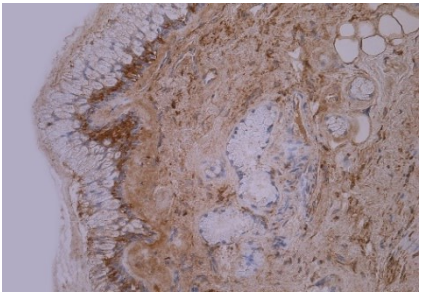

Figure 2.1. **Moderate (++) IL-1α immunoreactive cells in bronchial epithelium, few (+) – in mucosal connective tissue, as well as occasional (0/+) IL-1α positive cells in bronchial glands of 78-years-old male bronchial wall (COPD). IL-1α IHC, ×200**

Interleukin-4 (IL-4). In the bronchial epithelium of COPD patients (16), mostly numerous (+++) IL-4 immunoreactive cells were detected (Figure 2.2). Overall, most COPD patients (30) had few (+), few to moderate (+/++), moderate (++), and moderate to numerous (++/+++) IL-4 immunoreactive cells in mucosal connective tissue. In majority of COPD patients, occasional (0/+), few (+), few to moderate (+/++), and moderate (++) IL-4 positive cells were found in blood vessels (32), smooth muscle (37), and glands (23).

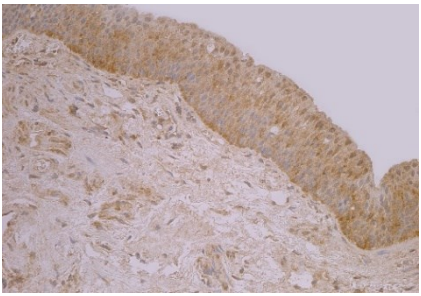

Figure 2.2. **Numerous (+++) IL-4 immunoreactive cells in bronchial epithelium together with metaplasia, as well as moderate (++) IL-4 positive cells in mucosal connective tissue of 60-years-old male bronchial wall (COPD). IL-4 IHC, ×200**

Interleukin-6 (IL-6). In all COPD patients, a variable number of IL-6-containing cells was found in the bronchial epithelium from none (0) to numerous, to abundant (+++/++++) (Figure 2.3). For most COPD patients, occasional (0/+) and few (+) IL-6 immunoreactive were detected cells in mucosal connective tissue (28), vascular wall (22), smooth muscle (31), and glands (15).

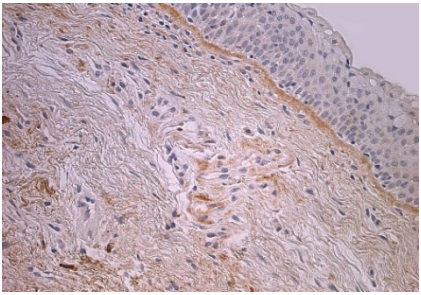

Figure 2.3. **None (0) of IL-6 immunoreactive cells in bronchial epithelium (with thickened basement membrane) and occasional (0/+) cells in mucosal connective tissue of 68-years-old male bronchial wall (COPD). IL-6 IHC, ×250**

Interleukin-8 (IL-8). In the bronchial epithelium of all COPD patients (40), a variable number of IL-8 immunoreactive cells from occasional (0/+) to abundance (++++) was found (Figure 2.4). In connective tissue (31) and blood vessels (36) of most COPD patients, few (+) to numerous (+++) IL-8-containing

cells were detected, but in smooth muscle (30) – occasional (0/+) to few (+), up to few to moderate (+/++).

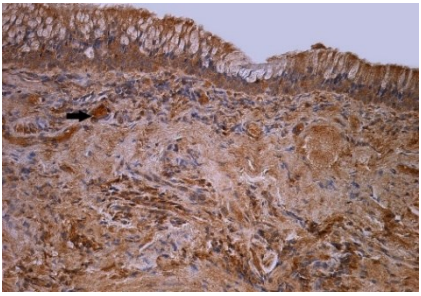

Figure 2.4. **Numerous (+++) IL-8-containing cells in epithelium, connective tissue and blood vessels (arrow) of 61-year-old man bronchial wall (COPD). IL-8 IHC, ×200**

Tumour necrosis factor-alpha (TNF-α). In part of COPD patients, moderate (++) TNF-α immunoreactive cells were detected in bronchial epithelium (11), connective tissue (14) and blood vessels (7), but in the rest of them – occasional (0/+) to numerous to abundance (+++/++++) of TNF-α positive cells (Figure 2.5). Occasional (0/+) and few (+) TNF-α immunoreactive cells were found in smooth muscle (29) and glands (23) of most COPD patients.

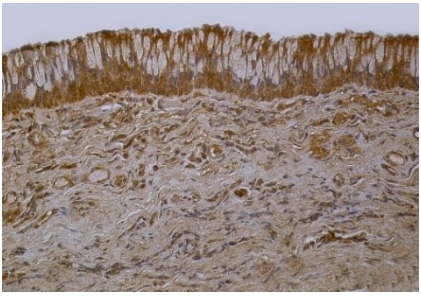

Figure 2.5. **Numerous to abundant (+++/++++) TNF-α immunoreactive cells in bronchial epithelium and numerous (+++) cells in mucosal connective tissue of 60-years-old male bronchial wall (COPD). TNF-α IHC, ×200**

Interleukin-7 (IL-7). In bronchial epithelium of COPD patients (13), mostly numerous to abundant (+++/++++) IL-7 immunoreactive cells were found (Figure 2.6), while in connective tissue (22) and blood vessels (18) – moderate to numerous (++/+++), to numerous (+++). In most COPD patients, few (+), few to moderate (+/++), moderate (++), and moderate to numerous (++/+++) IL-7-containing cells were determined in smooth muscle (28) and glands (16).

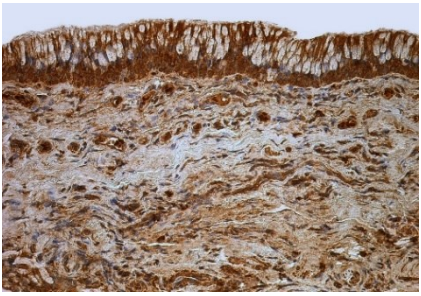

Figure 2.6. **Overall numerous to abundant (+++/++++) IL-7 immunoreactive cells in bronchial epithelium, mucosal connective tissue and blood vessels of 61-year-old male bronchial wall (COPD). IL-7 IHC, ×200**

Interleukin-12 (IL-12). In COPD patients, mostly moderate (++) to numerous (+++) IL-12 immunoreactive cells were found in bronchial epithelium (25), connective tissue (28) and blood vessels (29) (Figure 2.7), but in smooth muscle (20) and glands (16) – mostly few (+) IL-12-containing cells.

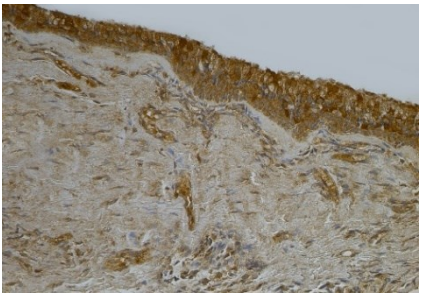

Figure 2.7. **Numerous (+++) IL-12 immunoreactive cells in bronchial epithelium, as well as moderate (++) numbers of cells in mucosal connective tissue and blood vessels of 60-years-old male bronchial wall (COPD). IL-12 IHC, ×200**

Interleukin-10 (IL-10). In most COPD patients, numerous (+++) to abundant (++++) IL-10 immunoreactive cells were found in bronchial epithelium (24), but moderate to numerous (++/+++), and numerous (+++) numbers of cells in conncective tissue (22) and blood vessels (17) (Figure 2.8). In all COPD patients, extensively variable number of IL-10 immunoreactive cells were determined in smooth muscle and glands from the deficiency (0) to abundance (++++).

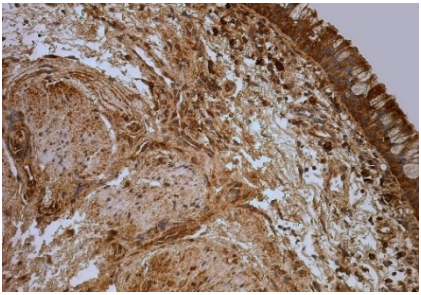

Figure 2.8. **Abundance (++++) of IL-10 immunoreactive cells in bronchial epithelium and connective tissue, also numerous (+++) immunoreactive cells in blood vessels and smooth muscle of 60-years-old male bronchial wall (COPD). IL-10 IHC, ×200**

Important factors in lung tissue remodelling

Transforming growth factor-β1 (TGF-β1). In all COPD patients, up to an abundance (++++) of TGF-β1-containing cells were found in the bronchial

epithelium, connective tissues and blood vessels (Figure 2.9). In most COPD patients, none (0) to moderate (++) TGF-β1 immunoreactive cells we detected in the bronchial smooth muscle (35) and glands (29).

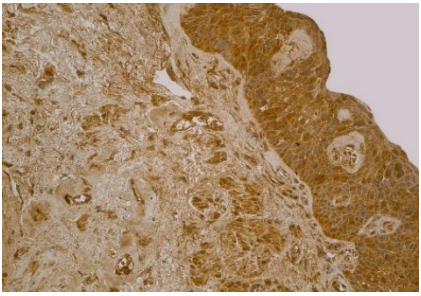

Figure 2.9. **Numerous to abundant (+++/++++) TGF-β1 immunoreactive cells in altered bronchial epithelium with metaplasia and mucosal connective tissue of 61-year-old male bronchial wall (COPD). TGF-β1 IHC, ×200**

Matrix metalloproteinase-2 (MMP-2). In all COPD patients, a highly variable number of MMP-2 immunoreactive cells were found in the bronchial epithelium up to an abundance (++++) (Figure 2.10), but in connective tissue and blood vessels – from their absence (0) to numerous (+++). In most patients, no (0) MMP-2 immunoreactive cell were detected in the bronchial smooth muscle (30) and glands (22), or those were found to be occasional (0/+) or few (+).

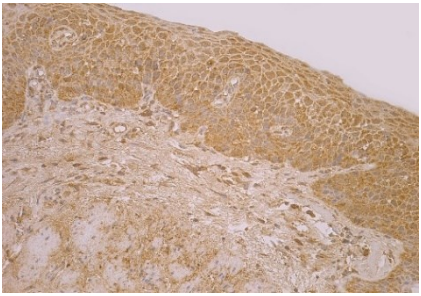

Figure 2.10. **Numerous to abundant (+++/++++) MMP-2 immunoreactive cells in altered bronchial epithelium with metaplasias and numerous (+++) cells in connective tissue, also few to moderate (+/++) cells in blood vessels and smooth muscle of 64-years-old male bronchial wall (COPD). MMP-2 IHC, ×200**

Tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). In most COPD patients, numerous (+++) and numerous to abundant (+++/++++) TIMP-2 immunoreactive cells were found in bronchial epithelium (22) (Figure 2.11), but in smooth muscle (17) and glands. (14) – occasional (0/+) and few (+). In all COPD patients, highly variable number of TIMP-2-containing cells were determined in connective tissue and blood vessels from their absence (0) to an abundance (++++).

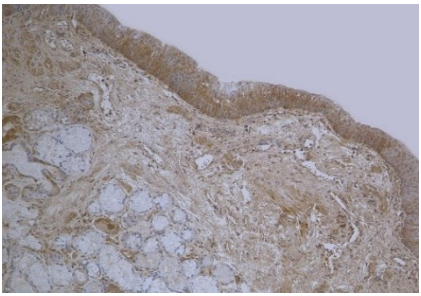

Figure 2.11. **Numerous (+++) TIMP-2 immunoreactive cells in bronchial epithelium, as well as moderate (++) cells in mucosal blood vessels and bronchial glands of 68-years-old male bronchial wall (COPD). TIMP-2 IHC, ×100**

Indicators of cellular and tissue oxidative stress

Heat shock protein-70 (Hsp-70). In most COPD patients, none (0) or occasional (0/+) Hsp-70 immunoreactive cells were found in bronchial epithelium (39), connective tissue (37), blood vessels (39), smooth muscle (36) and glands (32) (Figure 2.12).

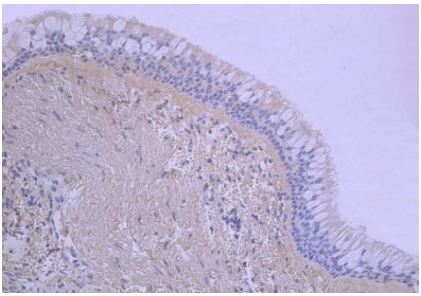

Figure 2.12. **Occasional (0/+) focal and weakly positive Hsp-70 immunoreactive cells in bronchial epithelium, but none (0) in mucosal connective tissue and blood vessels of 60-years-old male bronchial wall (COPD). Hsp-70 IHC, ×200**

Local antimicrobial protection factors

Human beta defensin-2 (hBD-2). In all COPD patients, variable amounts of hBD-2 immunoreactive cells were found in the bronchial epithelium, connective tissue and blood vessels from none (0) to numerous, to abundant (+++/++++) (Figure 2.13). In most COPD patients, none (0), occasional (0/+), few (+), few to moderate (+/++) hBD-2 immunoreactive cells were determined in smooth muscle (37) and glands (27).

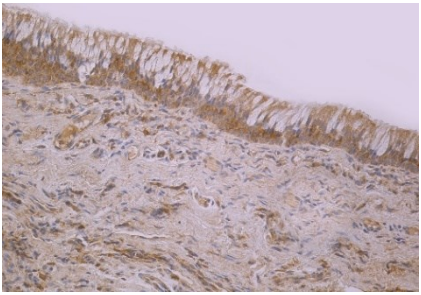

Figure 2.13. **Moderate to numerous (++/+++) hBD-2 immunoreactive cells in bronchial epithelium and mucosal connective tissue of 78-years-old male bronchial wall (COPD). hBD-2 IHC, ×200**

Human beta defensin-3 (hBD-3). In majority of COPD patients, occasional (0/+) to moderate (++) hBD-3 immunoreactive cells were found in bronchial

epithelium (24), connective tissue (30), and blood vessels (31) (Figure 2.14); however, in smooth muscle (33) and glands (24), none (0), occasional (0/+) and few (+) hBD-3 positive cells were determined.

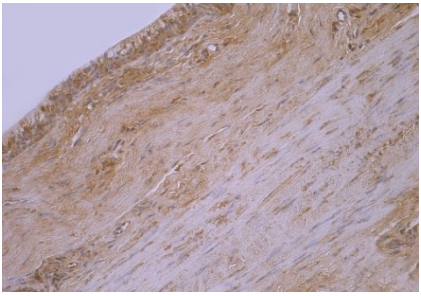

Figure 2.14. **Moderate (++) hBD-3 immunoreactive cells in bronchial epithelium, mucosal connective tissue, blood vessels and smooth muscle of 61-year-old male bronchial wall (COPD). hBD-3 IHC, ×200**

Human beta defensin-4 (hBD-4). In vast majority of COPD patients, either none (0) or occasional (0/+), or few (+) hBD-4 immunoreactive cells were detected in the bronchial epithelium (38), connective tissue (38), blood vessels (39), smooth muscle (39) and glands (33) (Figure 2.15).

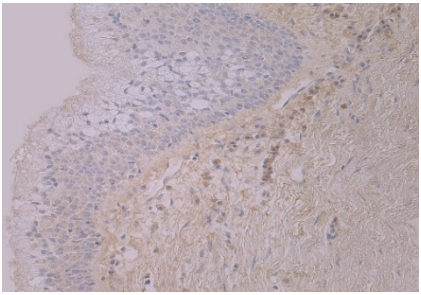

Figure 2.15. **None (0) hBD-4 immunoreactive cells in bronchial epithelium and blood vessels, but few (+) in mucosal connective tissue of 61-year-old male bronchial wall (COPD). hBD-4 IHC, ×400**

In general, smaller numbers of immunoreactive cells were determined in the bronchial smooth muscle and bronchial glands in the tissue material of COPD patients. Statistically significant ($p < 0.05$) lower numbers of **IL-7**, **IL-8**, **IL-10**, **IL-12**, **hBD-3** and **hBD-4** immunoreactive cells in bronchial smooth muscle should be highlighted in this finding.

Overall, more immunoreactive cells were determined in the bronchial epithelium, connective tissue and blood vessels of COPD patients. Apart from this finding, statistically significant ($p < 0.05$) higher numbers of **IL-1α**, **IL-4**, **IL-7**, **IL-8**, **IL-10**, **MMP-2** and **TIMP-2** immunoreactive cells in the bronchial epithelium should be highlighted (Table 2.2).

Evaluation of relationship between different tissue factors in COPD patients

In general, statistically significant ($p < 0.05$) correlations were determined between **IL-1α**, **IL-4**, **IL-7**, **IL-8**, **IL-10**, **TNF-α**, **MMP-2**, **TIMP-2**, **TGF-β1**, **hBD-2**, **hBD-3**, **hBD-4** immunoreactive cells in bronchial epithelium, connective tissue, blood vessels, smooth muscle and bronchial glands. The finding of statistically significant ($p < 0.05$) strong correlations between the numbers of **IL-8**, **IL-7**, **IL-10**, as well as **MMP-2**, **TIMP-2**, **TGF-β1** immunoreactive cells in different bronchial wall localisations should also be mentioned.

Table 2.2

Relative appearance and distribution of various tissue factors in lung tissues of COPD patients

	COPD patients (N = 40)				
	E.	**C.t.**	**B.v.**	**S.m.**	**G.**
IL-1α	++/+++ *	+/++	+	0/+	+
IL-4	+++ *	+/++ – ++	+/++	+	+/++
IL-6	+/++ – ++	0/+	+	0/+	0/+
IL-8	+++ *	++	++	+ *	+/++
TNF-α	++	++	++	+	+
IL-7	+++/++++ *	++/+++	++/+++	+/++ *	++
IL-12	++/+++	++/+++	++	+ *	+
IL-10	+++ *	++/+++	++/+++	+/++ *	+/++
TGF-β1	++/+++	++/+++	++ – ++/+++	+/++	+
MMP-2	++/+++ *	++	+/++	0/+	+
TIMP-2	+++ *	+/++ – ++	+/++	+	+
Hsp-70	0	0	0	0	0
hBD-2	+/++	++	+/++ – ++	+	+
hBD-3	++	++	+/++	0/+ *	+
hBD-4	0/+	0/+	0/+	0 *	0

Abbreviations in the table: E. – bronchial epithelium; C.t. – mucosal connective tissue; B.v. – mucosal blood vessels; S.m. – smooth muscle; G. – bronchial glands; C. – bronchial cartilage; A.e. – alveolar epithelium; A.m. – alveolar macrophages; IL-1α, -4, -6, -7, -8, -10, -12 – interleukin-1α, -4, -6, -7, -8, -10, -12; TNF-α – tumour necrosis factor-α; TGF-β1 – transforming growth factor-β1; MMP-2 – matrix metalloproteinase-2; TIMP-2 – tissue inhibitor of MMP-2; Hsp-70 – heat shock protein-70; hBD-2, -3, -4 – human beta defensin-2, -3, -4; 0 (0) – no immunoreactive cells (i.c.) in visual field (v.f.); 0/+ (0.5) – occasional i.c. in v.f.; + (1.0) – few i.c. in v.f.; +/++ (1.5) – few to moderate i.c. in v.f.; ++ (2.0) – moderate i.c. in v.f.; ++/+++ (2.5) – moderate to numerous i.c. in v.f.; +++ (3.0) – numerous i.c. in v.f.; +++/++++ (3.5) – numerous to abundant i.c. in v.f.; N – patient count; * – statistically significant difference ($p < 0.05$).

Evaluation of bronchoscopic examination data and immunohistochemical findings of different tissue factors in COPD patients

In COPD patients, bronchoscopically observed hypertrophy was associated with higher numbers of IL-8 immunoreactive cells in the bronchial epithelium ($U = 197.5$, $p = 0.037$) and glands ($U = 122$, $p = 0.041$), but lower numbers of hBD-3 immunoreactive cells in connective tissue ($U = 74.5$, $p = 0.026$) and blood vessels ($U = 76.5$, $p = 0.031$), as well as hBD-4-containing cells in the bronchial epithelium ($U = 73.5$, $p = 0.023$) and blood vessels ($U = 70$, $p = 0.017$). Also, chronic endobronchitis was associated with lower numbers of MMP-2 positive cells in the bronchial epithelium ($U = 79.5$, $p = 0.014$).

Evaluation of routine histology and immunohistochemical findings of various tissue factors in COPD patients

In COPD patients, the finding of bronchial epithelial metaplasia was associated with higher numbers of IL-6 immunoreactive cells in connective tissue ($U = 273.5$, $p = 0.044$), as well as lower numbers of IL-8 positive cells in the bronchial epithelium ($U = 122.5$, $p = 0.036$). Similarly, in COPD patients, the finding of fibrosis was associated with higher numbers of TNF-α immunoreactive cells in blood vessels ($U = 164$, $p = 0.017$), also MMP-2-containing cells in connective tissue ($U = 163.5$, $p = 0.017$) and blood vessels ($U = 168$, $p = 0.01$). Furthermore, vascular wall fibrosis was associated with higher numbers of IL-1α ($U = 280$, $p = 0.014$) and MMP-2 ($U = 282.5$, $p = 0.011$) immunoreactive cells in connective tissue, as well as IL-10 ($U = 277$, $p = 0.018$), MMP-2 ($U = 292$, $p = 0.005$), hBD-3 ($U = 283$, $p = 0.011$), and hBD-4 ($U = 272$, $p = 0.027$) positive cells in blood vessels. The granulation tissue finding in COPD patients was associated with lower numbers of IL-4 immunoreactive cells in the bronchial epithelium ($U = 87.5$, $p = 0.016$), as well

as IL-12 ($U = 99.5$, $p = 0.042$) and hBD- 2 ($U = 91.5$, $p = 0.022$) immunoreactive cells in blood vessels.

2.3 Comparison of the relative appearance and distribution of immunoreactive cells containing different tissue factors in the control group and COPD patients

Overall, a comparison of the relative appearance and distribution of immunoreactive cells containing different tissue factors in the control group and COPD patients showed statistically significantly ($p < 0.05$) more numbers of **IL-1α**, **IL-4**, **TNF-α**, **IL-7**, **IL-10**, **TGF-β1**, **TIMP-2** and **hBD-2** immunoreactive cells, as well as fewer numbers of **Hsp-70** immunoreactive cells in bronchial epithelium, mucosal connective tissue, blood vessels, bronchial smooth muscle and glands of COPD patients. Meanwhile, a comparison of the findings of different tissue factors in the control group and COPD patients indicated statistically significantly ($p < 0.05$) more numbers of **IL-6**, **IL-8**, **IL-12**, **MMP-2** and **hBD-3** immunoreactive cells, as well as fewer numbers of **IL-12** and **hBD-4** immunoreactive cells at various localisations in COPD tissue material. Moreover, in both COPD and control patients similar numbers of **IL-6**, **IL-8**, **hBD-3**, and **hBD-4** immunoreactive cells were determined at various localisations.

2.4 Analysis of various tissue factors in the ontogenesis perspective

2.4.1 Ontogenesis-related distribution of different tissue factors in the control group

- In all control patients, the numbers of **IL-4** immunoreactive cells in the bronchial epithelium and blood vessels, **IL-6**-containing cells in the bronchial epithelium, and **hBD-4** immunoreactive cells in the cartilage increased with ageing; however, the numbers of **hBD-4**-containing cells in the bronchial

epithelium and connective tissue, as well as **IL-6** immunoreactive alveolar macrophages decreased with ageing;

- in control patients aged 9–49 years, the numbers of **IL-8**, **IL-7**, **IL-10**, **hBD-4** immunoreactive cells in the bronchial epithelium, **hBD-3**-containing cells in connective tissue, **IL-8** and **IL-12**-containing cells in blood vessels, **IL-10** immunoreactive cells in smooth muscle, **IL-7**, **Hsp-70**, **hBD-3**-containing cells in cartilage, **IL-12** and **hBD-2** immunoreactive cells in alveolar epithelium decreased with ageing; in turn, the numbers of **TNF-α**-containing cells in smooth muscle, **IL-6** immunoreactive alveolar macrophages, as well as **hBD-4** immunoreactive cells in cartilage increased with ageing;
- in control patients aged ≥ 50 years, the numbers of **IL-1α** immunoreactive cells in bronchial epithelium, **TGF-β1**, **Hsp-70** immunoreactive cells in connective tissue, **TGF-β1**, **Hsp-70**, **hBD-2**, **hBD-4** immunoreactive cells in blood vessels, **TIMP-2**, **Hsp-70** immunoreactive cells in smooth muscle, **IL-12** immunoreactive cells in glands, **TNF-α** immunoreactive alveolar macrophages decreased with ageing; whereas, the numbers of **IL-4** and **IL-6** immunoreactive cells in the bronchial epithelium, also **IL-4** immunoreactive cells in the blood vessels increased with ageing.

2.4.2 Ontogenesis-related distribution of different tissue factors in COPD patients

Ontogenesis-related findings of medical history data in COPD patients

Overall, FEV_1 (%), FEV_1 (L), and FEV_1/FVC in subjects ≥ 75 years of age were on average lower compared to younger COPD patients.

Ontogenesis-related evaluation of the functional parameters and immunohistochemical findings of different tissue factors in COPD patients

Overall, the immunohistochemical findings of all tissue factors in COPD patients were not statistically significantly ($p \geq 0.05$) related to COPD duration,

severity, smoking history, and functional parameters (FEV_1 (L), FEV_1 (%), FVC (L), FVC (%), FEV_1/FVC).

Ontogenesis-related bronchoscopy findings in COPD patients

The presence of bronchoscopically evaluated chronic bronchitis was most commonly found in the bronchi of individuals aged 65–74 years (8) and ≥ 75 years (6), but was not detected in the bronchi of individuals aged 50–64 years. In addition, COPD patients with bronchoscopically diagnosed chronic bronchitis were statistically significantly older ($U = 92$, $p = 0.045$).

Ontogenesis-related routine histology findings in COPD patients

In COPD patients, a statistically significant difference was found between the age of the individuals and the epithelial metaplasia finding ($U = 115$, $p = 0.022$); that is, patients with epithelial metaplasia were statistically significantly older. Similarly, COPD patients with granulation tissue finding were more likely to be statistically significantly older ($U = 236$, $p = 0.045$).

Ontogenesis-related immunohistochemical findings of various tissue factors in COPD patients

- In all COPD patients, the numbers of **IL-6** immunoreactive cells in the mucosal connective tissue increased, but the numbers of **IL-8**-containing cells in the bronchial epithelium, blood vessels, smooth muscle, glands, as well as **IL-7**-containing cells in the blood vessels overall decreased with ageing;
- in COPD patients aged 50–64 years, the numbers of **hBD-4** immunoreactive cells in the bronchial epithelium and **IL-6**-containing cells in the bronchial glands increased with ageing;
- in COPD patients aged 50–64 years and 65–74 years, the numbers of **hBD-2**, **Hsp-70**-containing cells in bronchial epithelium, **IL-7**, **IL-8**, **Hsp-70**, **hBD-2**-containing cells in mucosal connective tissue, **IL-7**, **IL-8**, **hBD-2**,

hBD-3 immunoreactive cells in blood vessels, also **IL-8**, **TIMP-2** immunoreactive cells in smooth muscle decreased with ageing;

- in COPD patients aged 65–74 years, the numbers of **IL-4** immunoreactive cells increased, but the numbers of **TNF-α**-containing cells in smooth muscle decreased with ageing;
- in COPD patients aged 65–74 years and ≥ 75 years, the numbers of **IL-4**, **IL-6** immunoreactive cells in mucosal connective tissue, **IL-1α**-containing cells in mucosal blood vessels, **IL-1α**, **IL-4**, **IL-6**, **IL-12**, **hBD-4**-containing cells in smooth muscle, as well as **IL-6** immunoreactive cells in the bronchial glands increased with ageing;
- in elderly COPD patients aged ≥ 75 years, the numbers of **IL-1α** immunoreactive cells in smooth muscle, **IL-4**-containing cells in the bronchial epithelium, **MMP-2**-containing cells in the mucosal blood vessels, **IL-6**, **IL-10** immunoreactive cells in the bronchial glands increased, but the numbers of **IL-8** immunoreactive cells in bronchial epithelium, **IL-7**, **TGF-β1** immunoreactive cells in the bronchial glands decreased with ageing.

3 DISCUSSION

Routine histological findings of COPD-affected lung tissue

Routine histological findings of COPD-affected lung tissue showed significant changes in the epithelium, basement membrane, connective tissue, glands, smooth muscle, as well as blood vessels. Despite the specific function of each structure, all these changes can be assessed as non-specific and have been analysed separately in the works of many authors.

Although epithelial metaplasia is also reversible (Herfs et al., 2012), in the current study, the finding of epithelial metaplasia was statistically significantly associated with poorer and/or more variable functional parameters, as well as the severity of COPD. This is consistent with Rigden et al. (2016) study that found epithelial metaplasia in bronchial biopsies of people with COPD being associated with poorer functional parameters and severity of COPD (Rigden et al., 2016). Accordingly, the presence of bronchial epithelial metaplasia on routine histological examinations is associated with both a worse course of COPD and a less favourable prognosis.

In most COPD patients, fibrotic changes were found in the mucosal connective tissue with a large number of densely packed connective tissue collagen fiber bundles, between which rare connective tissue cells could be visualised. In some COPD patients, tissue bundles of collagen fibers were arranged chaotically and irregularly. Fibrotic changes in tissues are overall associated with chronic inflammation, in which tissue factors produced by immune (including CD4+ Th and CD8+ T lymphocytes, polymorphonuclear cells, B lymphocytes) and bronchial epithelial cells, and their interactions play an important role (Hogg, 2004; Yanagisawa et al., 2017). Fibrotic processes are one of the main factors leading to progressive airway obstruction in COPD (Chung, 2005). This indicates advanced remodelling processes, which are facilitated by e.g. worsening of COPD (Hogg et al., 2004). These data are

consistent with the statistically significant relationship between fibrotic findings and decreased functional parameters (FEV_1 (L), FEV_1 (%), FVC (L)) found in the current study; that is, the fibrosis finding was associated with poorer functional parameters. Patients with airway fibrosis are likely to have progressively increasing airway obstruction; however, progressive obstruction in COPD is caused not only by fibrosis, but also by epithelial metaplasia, mucoid epithelium, smooth muscle hyperplasia, bronchial gland hypertrophy, and thickening of bronchial wall due to the inflammatory infiltration (Chung, 2005).

Inflammatory cell infiltration was found in the bronchial epithelium, mucosal connective tissue, and bronchial glands of all COPD patients. Development of chronic inflammation and increase in the number of inflammatory cells (mainly macrophages, neutrophils, lymphocytes, eosinophils) locally in the bronchial wall is a classic sign of COPD (Barnes, 2014).

In COPD, chronic inflammation is characterised by marked activation and infiltration of immune cells into tissues, where innumerable signalling molecules interact, secreted by both immune and non-immune cells. (Taylor, 2010, Barnes, 2014). Elevated numbers of neutrophil and eosinophil leukocytes, macrophages, T and B lymphocytes, CD4+ and CD8+ T lymphocytes are typically found in tissue biopsies from COPD patients (Hogg et al., 2004; Barnes et al., 2006; Yoshida and Tuder, 2007; Brozyna et al., 2009). Neutrophils are one of the main inflammatory cells in the pathogenesis of COPD, and are closely related to the severity of COPD and the more unfavourable course of the disease (Singh et al., 2010). Presence of macrophages (Barnes, 2004a), dendritic cells (Givi et al., 2012), mast cells (Mortaz et al., 2011), and plasma cells (Zhu et al., 2007) in the course of chronic inflammation in COPD should also be mentioned.

In general, immune cells affect destruction and remodelling of the airways by directly secreting enzymes and inflammatory cytokines or by indirectly regulating the functions of other cells. Various factors secreted by immune cells

can promote airway extracellular matrix (ECM) destruction and remodelling processes, while other factors can protect against tissue damage and promote regeneration (Wang et al., 2018).

In the current study, inflammatory cell infiltrates were found in bronchial epithelium, mucosal connective tissue, and bronchial glands, confirming the involvement of inflammation in COPD morphopathogenesis. Infiltration of inflammatory cells in the bronchial epithelium, mucosal connective tissue, and bronchial glands of our patients ranged from mild to very pronounced, but, overall, it was more pronounced in mucosal connective tissue. The predominance of inflammatory cells in connective tissue is related to migration of inflammatory cells from the microcirculation blood vessels in the nearest connective tissue of the vascular basement membrane and endothelium, as well as to other sites in the bronchial wall, such as the epithelium and its surface (Rennard et al., 2007). Although identification of exact immune cells using immune cell-specific molecular markers was not performed, this direction would be an innovation in future research. The findings of various tissue factors described in the discussion below provides a sufficient basis for the overall discovery and presence of all these immune cells.

Statistically significant differences were found between hypertrophy observed during bronchoscopy and the degree of inflammatory cell infiltration in bronchial mucosal connective tissue in all the COPD patients, where, contrary to expectations, it was found that the hypertrophy was associated with lower inflammatory cell infiltration in mucosal connective tissue, also not with a smoking history and functional parameters of COPD patients. As bronchial wall thickening and further bronchial obstruction are also induced by other local changes (Chung, 2005), it should be assumed that the increase in tissue volume due to inflammatory cell infiltration is not so pronounced as to cause obstruction and corresponding spirographic findings alone; that is, obstruction is formed by the simultaneous combination of several morphological changes together.

In majority of COPD patients, mucosal connective tissue infiltration was determined together with granulation tissue, and the granulation tissue finding was statistically significantly associated with more pronounced inflammatory cell infiltration into mucosal connective tissue, as well as prolonged smoking (p/y). Granulation tissue formation in COPD is associated with changes in connective tissue and vascular remodelling following tissue damage under the influence of an irritant. Changes in vascularisation (as angiogenesis) and fibrosis (as the accumulation of ECM elements) contribute to remodelling of originally formed granulation tissue and scar tissue formation (Rennard et al., 2007), so the location of granulation tissue indicates a chronic course of processes.

Epithelial metaplasia and fibrosis are thought to be the most significant local changes with a direct effect on clinical COPD findings, airway obstruction manifestations and a less favourable prognosis for COPD. In contrast, mucoid epithelium, basement membrane thickening, bronchial gland hypertrophy, and smooth muscle hyperplasia could be associated with adaptive and compensatory local changes, regulatory function, and variability in COPD course and local changes.

Various tissue factors in relatively healthy lung tissue

In the lung tissues of the control group, **generally low levels of all tissue factors studied or IL-1α, IL-4, IL-6, IL-8, TNF-α, IL-7, IL-12, IL-10, TGF-β1, MMP-2, TIMP-2, Hsp-70, hBD-2, hBD-3, and hBD-4 immunoreactive cell counts at different localisations** were found. In general, the highest number of immunoreactive cells were located in the bronchial epithelium, bronchial cartilage, alveolar epithelium, and among alveolar macrophages of the control group patients.

Apart from these findings, statistically significantly higher numbers of IL-4, IL-7 immunoreactive cells in the bronchial epithelium, IL-8, TNF-α and TGF-β1 immunoreactive cells among alveolar macrophages, IL-8 immuno-

reactive cells in mucosal blood vessels and TNF-α immunoreactive cells in mucosal connective tissue must be noted compared to the presence of the same factor at other sites. Also, in the tissue material of the control group patients, fewer numbers of immunoreactive cells were determined in mucosal connective tissue, blood vessels, bronchial smooth muscle, glands. It can be agreed that such finding can be explained by the base or "baseline" level of various factors in the case of a relative norm and the potential variability in the case of different diseases (Rohmann et al., 2011). It should be emphasised that different cells in the lung tissue and their communication with others form and regulate different signaling pathways for maintenance of local immunity in a relative state of health (Vareille et al., 2011).

The concept of immunity has long been revised to take into account the potential ability of non-immune cells to secrete and respond to signalling molecules that are "typical" of immune responses, thus directly participating, for example, in the inflammatory process. Due to the continuous exposure to irritants and antigens, epithelial cells can activate local immunocompetent cells or induce their hemotaxis to the lesion site. Epithelial cells use autocrine and paracrine signalling to ensure efficient intercellular communication and its regulation, including with a feedback mechanism that to some extent reflects a peculiar homeostasis under relative normal conditions. A similar function has been observed for alveolar macrophages that are in direct contact with environmental factors in the alveolar part of the lung (Barnes, 2004a; Hallstrand et al., 2014).

Interestingly, a more pronounced finding of immunoreactive cells was determined also in bronchial cartilage of all the studied factors, except IL-4 and TNF-α, where the numbers of MMP-2, TIMP-2, hBD-2, hBD-3, hDB-4 immunoreactive cells was statistically significantly higher. There are practically no studies on the presence of various factors in the hyaline cartilage of the airways. In the case of chronic bronchitis, which is one of the manifestations of

COPD (Kim and Criner, 2013), chondrolytic processes have been identified, namely, more enzymes as well as degradation products have been found.

Inflammatory processes involving the involvement of mast cells and macrophages in the connective tissue adjacent to the hyaline cartilage were also observed, and these changes were not identified in the control hyaline cartilage (Tetlow et al., 1999). As in epithelium, for example, in cartilage under relative normal conditions, the presence of various factors could indicate a balanced "baseline" level of different signalling molecules, cartilage plasticity and the potential to participate in various tissue changes in COPD. However, no precise explanation of the moderate findings of some factors can be given yet, but relatively more pronounced findings of other factors in cartilage.

The presence of immunoreactive cells or even their absence in different localisations could be explained by the lower involvement of these structures in local signalling and their insignificance in intercellular communication under relative norm conditions.

Cytokines in COPD-affected lung tissue

In general, **the number of immunoreactive cells in bronchial epithelium, connective tissue and blood vessels was more pronounced** in the tissue material of COPD patients. Of all the tissue factors studied, **numbers of immunoreactive cells for IL-1α, IL-4, IL-8, IL-7, IL-12, IL-10, TGF-β1, MMP-2 and TIMP-2 were among the most pronounced in the bronchial epithelium, IL-8, TNF-α, IL-7, IL-12, IL-10, TGF-β1, MMP-2, hBD-2 and hBD-3 – in mucosal connective tissue, while IL-8, TNF-α, IL-7, IL -12, IL-10, TGF-β1 – in blood vessels**, compared to the presence of the same factors in other localisations. Apart from these findings, **a statistically significant increased numbers of IL-1α, IL-4, IL-7, IL-8, IL-10, MMP-2 and TIMP-2 immunoreactive cells in the bronchial epithelium** should be highlighted. In turn, less numbers of immunoreactive cells were determined in bronchial smooth

muscle and bronchial glands in the tissue material of COPD patients; moreover, statistically significantly less number of IL-7, IL-8, IL-10, IL-12, hBD-3 and hBD-4 immunoreactive cells in bronchial smooth muscle.

Before discussing the role of each cytokine in the morphopathogenesis of COPD, it is important to emphasise the common role of cytokines. Hereby, inflammatory cytokines (such as IL-8 and TNF-α) are actively secreted by both immune and bronchial wall tissue cells (such as neutrophils, tissue macrophages, bronchial epithelial cells), which together organise inflammatory processes (Barnes, 2004b). The heat shock protein Hsp-70 released during cell damage further stimulates the release of IL-6 and IL-8 by linking cell and tissue damage to the inflammatory process (Sarir et al., 2008). The pathogenesis of COPD is associated with IL-1 (IL-1α, IL-1β) (Hulina-Tomašković et al., 2019), IL-6, IL-8 (Gabryelska et al., 2019), IL-4, IL-7 (Perotin et al., 2014; Wu et al., 2014, Shibata et al., 2018), IL-10 (Wei and Sheng Li, 2018), IL-12 (Castellucci et al., 2015) and TNF-α (Zhou et al., 2018) local secretion into tissues, which determines chronic and continuous inflammation in COPD (Bai et al., 2019).

Unambiguously, all locally secreted tissue factors affect the immune cell profile of COPD. Of particular importance, the involvement of non-immune cells (epithelial cells, fibroblasts) in intercellular communication networks, as well as the molecular mechanisms and signalling that further influence morphological changes in COPD must be highlighted.

In general, COPD is thought to be associated with various/large numbers of cytokines and their interactions, and to date, locally elevated levels of IL-1, IL-4, IL-6, IL-7, IL-8, IL -12, TNF-α have been identified in COPD-affected tissues (Caramori et al., 2016; Cho et al., 2019). Studies have shown that Th1-associated cytokines IL-1, IL-8, IL-12 and TNF-α, as well as Th2-associated cytokines IL-6 and IL-10 are linked with COPD (Baines et al., 2011).

In COPD patients, most **IL-1α** immunoreactive cells were detected in bronchial connective tissue and bronchial epithelium; moreover, the statistically

significant more pronounced numbers of IL-1α immunoreactive cells in the epithelium were found. The findings of IL-1α at different localisations in COPD patients were statistically significantly associated with the findings of IL-4, IL-6, IL-10, MMP-2, hBD-2, and hBD-3, especially in the bronchial epithelium and glands. Because IL-1 family factors (including IL-1α) are associated with innate and acquired immune functions, and IL-1α is one of the major inflammatory cytokines (Garlanda et al., 2013), the presence of IL-1α in control tissues can be assessed as a slight sign of an inflammatory response under physiological normal conditions to persistent and continuous contact with the environment, which is also associated with the interaction of cytokines IL-4, IL-6, IL-7 and IL-10. Although IL-1α is known to regulate various cytokines involved in the inflammatory process (i.e., not just inflammatory cytokines), other cytokines also regulate IL-1α secretion. Thus, it is known that under normal conditions, epithelial cells both secrete IL-1α, IL-4, IL-6, IL-10 and receive signals inducing these factors (Vareille et al., 2011; Hallstrand et al., 2014).

Respiratory epithelial cells are being studied as a source of IL-1α cytokine because the IL-1α precursor is known to be located in the respiratory epithelium (Iwasaki and Medzhitov, 2015). Once IL-1α is produced in epithelial cells, the production of other cytokines is subsequently observed. It is thought that the causal regulation of inter-regulatory cytokines results in the release of IL-1α first, followed only by the release of other factors. IL-1α is associated with the ability to initiate an inflammatory response, as well as to provide further maintenance and enhancement of inflammation in COPD, where bronchial epithelium is named as one of the major sites of IL-1α production. Further release of IL-1α from also other cells may initiate a cascade of various cytokine signalling and initiate or maintain an inflammatory process, including chronic inflammation over a long period of time if the irritant effect is not eliminated (Garlanda et al., 2013).

Respiratory epithelial cell “COPD phenotype” has been reported to be associated with the ability of epithelial cells to release various cytokines or, conversely, to limit their release (Nadigel et al., 2013). Respiratory epithelial cells activate and further release various cytokines such as IL-1α, IL-4, IL-6, IL-8, and IL-10 (Vareille et al., 2011; Hallstrand et al., 2014). In addition, epithelial cells are able to produce inflammatory, anti-inflammatory and regulatory mediators such as IL-1, IL-6, IL-8, TNF-α in response to various stimuli (Barnes, 2018; Zeng et al., 2018).

This finding suggests that IL-1α is considered to be one of the earliest mediators of inflammation in COPD and thus IL-1α is associated with the initiation, maintenance and enhancement of a complex inflammatory process in COPD with the most prominent role of airway epithelium in these processes. In COPD patients, we also found a statistically significantly higher numbers of IL-1α immunoreactive cells in mucosal connective tissue associated with a thickened microcirculatory vessel wall. This is partly consistent with the findings of other researchers, namely that IL-1α is associated with the formation of fibrosis sequentially after acute inflammation (Borthwick, 2016).

Overall, in COPD patients, the highest number of **IL-4** immunoreactive cells was detected in the bronchial epithelium; moreover, the numbers of IL-1α immunoreactive cells in the epithelium were statistically significantly more pronounced. The finding of IL-4 at different localisations in COPD patients was generally statistically significantly associated with IL-1α, IL-6, IL-8, IL-10, TNF-α, TGF-β1, MMP-2, TIMP-2, hBD-2, as well as the presence of hBD-3 in various localisations, especially in the bronchial epithelium and glands.

Because this finding was similar with IL-1α, it has been hypothesised that IL-4 performs a number of important functions in COPD. Thus, IL-4 increases fibroblast proliferation and the production of ECM components that further promote fibrosis (Luzina et al., 2015). IL-4 signalling is associated with the pathogenesis of COPD, in which IL-4 production is observed from a large

number of immune cells. The main sources of IL-4 production are Th2 cells, mast cells, basophils, and eosinophils; however, the main functions are related to increased formation of eosinophils and Th2 lymphocytes, but decreased formation of Th1 lymphocytes, increase in IgE production, as well as more pronounced mucin production and increase in goblet cell count (Barnes et al., 2009).

Similar function has been observed for CD8+ T lymphocytes whose secreted IL-4 promotes mucus hypersecretion as a characteristic of chronic bronchitis (Miotto et al., 2003). IL-4 also promotes differentiation of T lymphocytes themselves (Barnes et al., 2009), affects T lymphocyte function, causes bronchial hyperplasia and mucus overproduction, as well as smooth muscle cell hyperplasia and hypertrophy (Kumar and Sharma, 2010). Although no statistically significant association of IL-4 with these tissue changes was established in the current study, it is likely that IL-4 may be present in part to the aforementioned morphological changes; however, to an individual extent and in the presence of other molecular events. Similar presence of IL-1α and IL-4 in alveolar macrophages of control group lung tissue, but in bronchial epithelium of COPD patients, suggests involvement of airway parts in response to irritants in normal and diseased cases, as well as the fixed interaction of IL-1α and IL-4.

In COPD patients, most **IL-6** immunoreactive cells were detected in mucosal blood vessels and bronchial epithelium, and the finding in bronchial epithelium was statistically significantly the most pronounced. In the tissues of COPD patients, the IL-6 findings were statistically significantly related to the IL-8, IL-10, and TIMP-2 findings. Knowing that IL-6 induces the release of other cytokines, it has been assumed that this function is relevant in all of the above mentioned localisations.

However, regenerative and protective functions of IL-6 have also been studied (Scheller et al., 2011). IL-6 functions are co-dependent on localisation of other tissue factors locally in tissues, where in the presence of many factors

IL-6 can perform diametrically opposite functions; that is, acting as an inflammatory and anti-inflammatory cytokine (Barnes, 2004b). Furthermore, IL-6 is a pleiotropic cytokine that controls T cell infiltration in the lung, promotes mucus secretion, promotes mast cell proliferation and contractions in bronchial smooth muscle cells (Xia et al., 2013; Luzina et al., 2015). It is important that IL-6 regulates the regulatory function of other cells, as well as realises the immunomodulatory function, including in smooth muscle (Xia et al., 2013). IL-6 is secreted early from healthy cells compared to infected cells during the infection, linking this finding to the regulatory potential of IL-6 (Copenhaver et al., 2015).

The main sources of IL-6 are structures that are more susceptible to airborne antigens; however, IL-6 is intensively secreted by healthy cells rather than by irritant-damaged cells. Therefore, the functions of IL-6 could be explained by the mediator role of IL-6 in various signaling pathways rather than initiating inflammatory responses. In COPD patients, statistically significantly more pronounced numbers of IL-6 immunoreactive cells were also found in mucosal connective tissue associated with bronchial epithelial metaplasia, suggesting increased IL-6 production in connective tissue during epithelial damage, most likely due to other factors released by metaplasic epithelial cells.

In COPD patients, more **IL-8** immunoreactive cells were detected in the bronchial epithelium, which was also a statistically significant finding. The numbers of IL-8 immunoreactive cells were statistically significantly associated with the findings of TNF-α, IL-7, IL-10, TGF-β1, MMP-2, TIMP-2, and hBD-2 immunoreactive cells at various localisations. This relationship was particularly pronounced between IL-8 and IL-7, IL-10, and TIMP-2 at different localisations.

The cytokine IL-8 is commonly involved in inflammatory processes associated with the involvement of neutrophils (Barnes, 2001). In general, in COPD-affected lung tissue, IL-8 is produced in the lung by neutrophils, T lymphocytes, monocytes, macrophages (including tissue macrophages),

NK cells and mast cells (Mukaida, 2003), as well as airway epithelial cells, fibroblasts, endothelocytes, and smooth muscle cells (van Eeden and Terashima, 2000; Mukaida, 2003; de Boer et al., 2007; Sarir et al., 2008), suggesting that this cytokine is directly involved in the functions of these structures under inflammatory conditions. Also, one of the major sources of inflammatory cytokines IL-8 is the airway epithelium (Sarir et al., 2008; Nadigel et al., 2013), which explains markedly more IL-8 immunoreactive cells in the bronchial epithelium that has been detected.

When studying the association of the IL-8 with IL-7 in COPD patients, there is practically no literature on the interaction of these cytokines. IL-8 in the lung induces hematopoiesis, hematopoietic cell mobilisation and activates the monocyte-macrophage system, provides leukocyte and macrophage hematotaxis and activation, coordinates cell migration, initiates inflammation with neutrophil sequestration, extravasation, induces leukocyte degranulation and increases vascular permeability (van Eeden and Terashima, 2000; Mukaida, 2003; Sarir et al., 2008; Allen and Kurdowska, 2014).

Considering the similar regulatory role of IL-7 (Dooms, 2013; Zaunders et al., 2014), the association between IL-8 and IL-7 could be related to the maintenance of the inflammatory response and the regulation of inflammatory cell development, differentiation, and functionality. In turn, IL-10 directly inhibits the secretion of IL-8 from immune cells (Taylor et al., 2007, Commins et al., 2008, Castellucci et al., 2015, Higaki et al., 2015); therefore, it is believed that IL-10 inhibits the effects of IL-8 and normalises inflammatory processes, thus reducing potential tissue damage.

IL-8-activated neutrophils soon degranulate, releasing proteases that further cause epithelial, ECM damage, and airway remodelling. Simultaneously, growth factors are released, which act in the opposite direction or promote tissue regeneration. The balance of these processes is determined by the distribution of other factors in the tissues, which in turn determines either the process of

destruction or the predominance of regeneration processes (de Boer et al., 2007). Like IL-10, TIMP-2 could have a similar, i.e. inhibitory, effect on IL-8, which could explain the close relationship between IL-8 and TIMP-2. In COPD patients, statistically significantly higher numbers of IL-8 immunoreactive cells were found in the bronchial epithelium and glands associated with bronchoscopically determined hypertrophy. It is possible that TIMP-2, by inhibiting the regulation of IL-8, simultaneously promotes the formation of fibrosis, which is one of the main functions of TIMP-2 (Arpino et al., 2015).

In COPD patients, statistically significantly lower numbers of IL-8 immunoreactive cells were also found in the bronchial epithelium and glands associated with bronchial epithelial metaplasia. In healthy individuals, airway epithelial cells secreted more IL-8 in immediate response to cigarette smoke, while airway epithelial cells in COPD-affected individuals secreted less IL-8 (Nadigel et al., 2013). This could indicate airway epithelial cell dysfunction in epithelial damage, inflammatory process and subsequent epithelial metaplasia.

In COPD patients, most **TNF-α** immunoreactive cells were detected in connective tissue and bronchial epithelium, as well as in blood vessels. In the tissues of COPD patients, the presence of TNF-α was statistically significantly related to the presence of MMP-2, TIMP-2, hBD-2, hBD-3, and hBD-4 in different localisations, whereas in control goup – also to IL-10.

TNF-α is one of the indicators of the "depth" of inflammation that apparently affects the bronchial mucosa, especially connective tissue, in the studied COPD patients. TNF-α, released by airway epithelial cells and macrophages, initiates inflammatory processes and is one of the fastest secreted inflammatory cytokines (Brusselle et al., 2011), in addition, TNF-α induces the release of other inflammatory cytokines that together have broad inflammatory properties (van den Berg et al., 2005; Sarir et al., 2008).

Due to stimulation of inflammatory cytokines (examples given, IL-6, IL-8, TNF-α), various immune cells participate in inflammatory responses, which are subsequently associated with further airway wall remodelling (Srivastava et al., 2007). In the experimental COPD model, increased expression of IL-6 and TNF-α was detected in the lungs of animals compared to controls (Li et al., 2016). High levels of IL-8 and TNF-α together in the lung tissue of COPD patients may indicate an excessive inflammatory response to irritant-induced tissue damage. Unfortunately, in an inflammatory environment, TNF-α escapes the physiologically inhibitory effects of IL-10 (Castellucci et al., 2015). This could explain why the association of TNF-α finding was determined with IL-10 in the tissues of the control group but not in the tissues of COPD patients.

In COPD patients, statistically significantly higher numbers of TNF-α immunoreactive cells were also found in the mucosal blood vessels associated with the finding of fibrosis. TNF-α is known to induce the release of MMP-2 in connective tissues of COPD-affected tissues (Lagente et al., 2005), but MMP-2 release is also closely related to TIMP-2 release in maintaining tissue remodelling balance (Rohani and Parks, 2015). TGF-β1 exposed to TNF-α reduces the synthesis of active MMP-2 and total MMP-2 in lung fibroblasts, suggesting that TGF-β1, MMP-2, TIMP-2 and TNF-α may be interrelated in pulmonary inflammation and fibrosis (Ye et al., 2011). Because the association of TNF-α was determined with MMP-2 and TIMP-2 in COPD patients, it is thought that the presence of TNF-α and fibrosis is determined by the interactions of MMP-2, TIMP-2 and TGF-β1 altogether, and TNF-α is involved in remodelling.

The release of hBD-2 (Li et al., 2015) and hBD-3 (Dhople et al., 2006) in airway epithelial cell culture is known to be mediated by TNF-α; therefore, the presence of TNF-α in both control and COPD patients is associated with hBD-2, hBD-3; hBD-4 finding could be related to the regulation of antimicrobial protection under inflammatory conditions. Although TNF-α does not affect

hBD-4 expression, it is thought that this finding could be explained by the structural and functional association of hBD-4 with other defensins (García et al., 2001a; Jarczak et al., 2013).

In COPD patients, most **IL-7** immunoreactive cells were also detected in the bronchial epithelium, and this finding was statistically significant. Relative numbers and appearance of IL-7 immunoreactive cells in different localisations of COPD patients was statistically significantly related to findings of IL-10, TGF-β1, MMP-2, and TIMP-2. The previously discussed association of IL-8 and IL-7 with the inflammatory process and cellular regulation, which could explain the interaction between IL-7 and IL-8, could also mark the involvement of IL-10 in this union, as IL-8 is closely associated with IL-10. Unfortunately, a uniform explanation for the relationship between IL-7, IL-8, and IL-10 in the literature was not found; however, it is assumed that all these cytokines are associated with T lymphocyte subpopulations (Allen and Kurdowska, 2014; Zaunders et al., 2014; Castellucci et al., 2015; Higaki et al., 2015; Akdis et al., 2016).

Most **IL-12** immunoreactive cells were detected in blood vessels, connective tissue, and bronchial epithelium of COPD-affected tissue. Here, IL-12 findings were related to the IL-10, Hsp-70 and hBD-3 findings. In mouse lung tissue, IL-12 promotes remodelling processes and fibrosis, as well as induces TGF-β1 gene expression and tissue production (Hackett et al., 2014); therefore, it is thought that IL-12 function in the lung tissues of both healthy and diseased individuals could be associated with regeneration. In contrast, the interaction between IL-12 and hBD-3 suggests that both factors are linked by immune cell regulatory functions (Nagaoka et al., 2008; Commins et al., 2010; Haarmann et al., 2015). The interaction between IL-12 and IL-10 can be explained by the inhibitory effect of IL-10 on the release of IL-12 from immune cells, and IL-10 inhibits IL-12 via macrophages (Taylor et al., 2007; Commins et al., 2008; Higaki et al., 2015). These findings can be considered as evidence of the broad functions of IL-12. Hereby, the role of IL-12 in the

morphopathogenesis of COPD, for which there are no practical data in the literature, is considered to be innovative and original.

Overall, in COPD patients, most **IL-10** immunoreactive cells were detected in connective tissue, blood vessels, and bronchial epithelium, where statistically significantly most pronounced findings were estimated in bronchial epithelium. In addition to the above mentioned association of IL-10 with IL-1α, IL-4, IL-6, IL-8, IL-7 and IL-12 in the tissues of COPD patients, the association with MMP-2, TIMP-2, TGF-β1 must also be highlighted. The anti-inflammatory response with co-existing inflammation reveals intercellular signalling of inflammatory, anti-inflammatory and regulatory cytokines, suggesting that cells capable of secreting IL-10 have a strong influence on the control of the immune response over inflammation (examples given, intensity, localisation, extent, duration) (Akdis et al., 2016; Barnes, 2016; Wang et al., 2018). IL-10 is a potent anti-inflammatory cytokine where the release of IL-10 maintains an inhibitory effect on inflammatory processes (Brusselle et al., 2011). IL-10 inhibits inflammatory cytokines IL-1, IL-4, IL-6, IL-8, IL-12 and TNF-α (Taylor et al., 2007; Commins et al., 2008; Castellucci et al., 2015; Higaki et al., 2015), which corresponds to the findings of the current study.

Accordingly, the role of IL-10 in the maintenance of the anti-inflammatory response in both acute and chronic inflammatory conditions should be highlighted in the pathogenesis of COPD. It should be emphasised that in the general context of cytokine interactions and local tissue changes in COPD-affected lung tissue, IL-10 provides a local protective function by stabilising inflammatory processes, cell and tissue damage (Nadigel et al., 2013). It is possible that this explanation also partially substantiates the association of the IL-10 with MMP-2, TIMP-2, TGF-β1 in COPD patients' tissues, although the association of the IL-10 with these remodelling factors was practically not found in the literature. In vitro animal experiments have shown that recombinant IL-10 promotes TGF-β1 expression in alveolar macrophages (Barbarin et al.,

2004). IL-10 is thought to be directly associated with TGF-β1, which in turn indirectly links IL-10 to MMP-2 and TIMP-2, and to remodelling events induced by both factors.

Remodelling factors and activity indicators in COPD-affected lung tissue

Tissue remodelling processes are significantly influenced by one of the most powerful and multifunctional molecules, TGF-β1. This factor regulates immune function, inflammatory processes, tissue remodelling, fibrosis, as well as wound healing (regeneration). These multifunctional properties are, of course, variable when TGF-β1 is present in the environment of other molecules (growth, inflammatory, anti-inflammatory, regulatory) (Tang et al., 2014).

The overall context of all ongoing tissue changes should be considered when determining the effects of TGF-β1 (Stewart et al., 2018). In tissue damage caused by oxidative stress and inflammatory reactions, ECM degradation processes occur. Peptidases of tissue structural components (example given, MMP-2) degrade ECM collagen, elastin, basement membrane, laminin, and fibronectin. Interestingly, broken matrix components can further enhance inflammatory processes (Saito et al., 2018). TIMP-2 is a critical MMP-2 antagonist that promotes tissue healing and wound healing, and TIMP-2 is associated with better airway functional finding in COPD (Turino, 2007; Ghanei et al., 2010).

In general, in COPD patients, most **TGF-β1** immunoreactive structures were detected in blood vessels, connective tissues, and bronchial epithelium, which means that these structures are intensively remodelled in COPD. This is also confirmed by the fact that in COPD patients, statistically significant relationship between TGF-β1 and MMP-2, TIMP-2 immunoreactive cell count was found. TGF-β1 secretion from damaged epithelial cells induces regeneration of airway epithelium and further ECM remodelling in mucosal connective tissue (Boxall et al., 2006), promotes cell proliferation, metaplasia, and airway fibrosis

(Shaykhiev and Crystal, 2014). In human lung tissue fibroblast cultures, TGF-β1 inhibits the secretion of both MMP-2 and TIMP-2 by balancing remodelling processes (Eickelberg et al., 1999).

Although TGF-β1 is associated with broad protective functions by preventing adverse morphopathogenic processes, such as the onset of recurrence of epithelial damage in epithelial cell culture, the presence of other factors may unfortunately reduce the effects of TGF-β1, namely MMP-2 promotes epithelial damage (Lechapt-Zalcman et al., 2006). TGF-β1 in the presence of the cytokine TNF-α caused regulation of MMP-2 production in lung fibroblasts; that is, by reducing the active form of MMP-2 and the overall synthesis of MMP-2 (Ye et al., 2011), suggesting that TGF-β1, MMP-2 and TNF-α may have a common association in inflammation and fibrosis. Since TNF-α promotes the release of MMP-2 (Lagente et al., 2005), it is thought that TGF-β1 already indirectly inhibits both ECM degradation processes and fibrosis during inflammation well before remodelling processes begin. This is a very important protective function of TGF-β1 in balancing remodelling processes. In general, TGF-β1 is an important participant in COPD morphopathogenesis, which is involved in both inflammatory and remodelling regulatory functions.

In COPD patients, most **MMP-2** immunoreactive cells were detected in blood vessels, connective tissue, and bronchial epithelium, and the increased amount of MMP-2 immunoreactive cells in bronchial epithelium was statistically significant. In practice, it can be concluded that bronchial wall structures actively secrete MMP-2. In COPD patients, a statistically significant relationship was determined between MMP-2 and TIMP-2, hBD-2. Overall, MMP-2 is found in bronchial epithelial cells, endothelial cells, macrophages, pneumocytes, fibroblasts, and smooth muscle cells (Hayashi et al., 1996; Kawano et al., 1997; Solli et al., 2013; Zou et al., 2016).

Epithelial tissue is thought to be the major source and store of MMP-2, not connective tissue (Xu et al., 2002). In COPD, inhaled airborne irritants can

damage epithelial power, and this damage can further provoke inflammation by attaching new immune cells and activating the proteolytic environment, mainly through the use of collagenase MMP-2 (Bagdonas et al., 2015). This is consistent with the finding of the present study that the MMP-2 is more pronounced in epithelial tissue. In COPD patients, higher levels of MMP-2-containing cells were found in epithelial and connective tissues, which is thought to be associated with the highlighted remodelling processes in these localisations. MMPs produced in epithelial tissues may affect remodelling of lung tissue distantly in subepithelial connective tissue. Activated bronchial epithelial cells modify the airway microenvironment by autocrine release of both pro-MMP-2 and active MMP-2 (Chu et al., 2006).

In COPD patients, a statistically significantly lower finding of MMP-2 immunoreactive cells was also found in the bronchial epithelium associated with bronchoscopically determined chronic endobronchitis. Although COPD is classically associated with a pronounced MMP-2 finding, the opposite data are also found in the literature, namely, MMPs in human airway epithelial cells were found in only a few lung epithelial cells in control subjects (Miller et al., 2008), as well as the release of MMP-2 from connective tissue fibroblasts decreases under the influence of cigarette smoke irritant (La Rocca et al., 2007). This suggests the regulation of MMP-2 by various other factors, as well as the known variability and individual course of both MMP-2 findings and morpho-pathogenesis processes.

In COPD patients, a statistically significant increase in the presence of MMP-2-containing cells was found in the mucosal connective tissue and blood vessels associated with the fibrosis finding, as well as with the thickened vessel wall. This finding is related to the balance of MMP-2 and TIMP-2. In the case of fibrosis, since TIMP-2 predominates over MMP-2, the amount of MMP-2 may increase too in an effort to maintain the balance of remodelling processes (Sariahmetoglu et al., 2007; Rohani and Parks, 2015).

In COPD patients, most **TIMP-2** immunoreactive cells were detected in blood vessels, connective tissue, and bronchial epithelium, and TIMP-2-containing cells were statistically significantly more pronounced in bronchial epithelium. Also, TIMP-2 was statistically significantly associated with hBD-2, hBD-3. Interestingly, the number of TIMP-2 immunoreactive cells in the bronchial epithelium, blood vessels and smooth muscle was higher than the number of MMP-2 immunoreactive cells, but similar or not significantly lower in the bronchial glands and mucosal connective tissue, which means that TIMP-2 does not inhibit tissue degradation in stated localisations. TIMP-2 is found in epithelial cells, macrophages, alveolar epithelium, fibroblasts, and normal lung tissue myofibroblasts (Hayashi et al., 1996; Karakiulakis et al., 2007).

The distribution model of TIMP-2 and MMP-2 suggests that the effects of TIMP-2 are primarily local and often correspond to the location of MMP-2. To maintain the environmental stability of the ECM, TIMP-2 directly inhibits MMP-2, so the location of the TIMP-2 is critical. TIMP-2 is thought to directly inhibit MMP-2, further inhibiting TGF-β1; moreover, when activated by MMP-2, TGF-β1 is secreted in an enhanced manner to increase fibrous activity in connective tissue stromal cells and could cause fibrosis (that is, in contrast to the effect of MMP-2). Because TIMP-2 already inhibits MMP-2, TGF-β1 activation is limited, and ECM deposition may be reduced accordingly (Arpino et al., 2015). Therefore, the discovered increase in TIMP-2 was a response to increased MMP-2 and TGF-β1. Presumably, the major tissue site in COPD remodelling is associated with the predominance of TIMP-2 over MMP-2, and this hypothesis is supported by the site of airway fibrosis in the study patients.

In general, very few or no **Hsp-70** immunoreactive cells were detected in COPD patients at all localisations, indicating reduced cell activity. The synthesis of Hsp-70 is enhanced during regulated physical or chemical stress, so it helps prevent protein denaturation and degradation to protect all human tissues against cell and tissue damage (Lee et al., 2004; Qu et al., 2015). Although Hsp-70 plays

a protective role against cytotoxic lesions and is regulated by a variety of factors, significant adaptation to chronic oxidative stress occurs by lowering Hsp-70 levels (Xie et al., 2010; Qu et al., 2015). The low numbers of Hsp-70 immunoreactive cells estimated in the current study could occur if stress conditions are prolonged, and continuous exposure to the irritant maintain cell and tissue damage, which may lead to Hsp-70 depletion. This is also supported by the high level of Hsp-70 described in the literature in the case of acute damage (Qu et al., 2015), but low – as a result of prolonged irritant exposure (Wu et al., 2013).

Hsp-70, which is secreted by the body's own cells, acts on bell-like receptors (TLRs) in a similar way to molecules of bacterial origin, activating the release of some defensins, for example, bell-like receptor-2 (TLR-2) is known to regulate hBD-2 secretion (Bals and Hiemstra, 2004), which could explain why the association of Hsp-70 with beta defensins was determined.

Local antimicrobial protection in COPD-affected lung tissue

In COPD patients, most **hBD-2** immunoreactive cells were detected in bronchial epithelium, blood vessels, and connective tissue. In the control group and COPD patients, a statistically significant relationship between hBD-2 and hBD-3, hBD-4 findings was determined in different localisations. In COPD patients, a statistically significant increase in numbers of hBD-2 immunoreactive cells was also found in mucosal connective tissue in association with thickened basement membrane, which could indicate epithelial-connective tissue interactions, as well as the mediated role of hBD-2 in remodelling; however, the literature data is poor on this topic.

In COPD patients, most **hBD-3** immunoreactive cells were detected in blood vessels, connective tissues, and bronchial epithelium. In contrast, in COPD patients, few **hBD-4** immunoreactive cells were detected at all localisations

overall. Tissue antimicrobial protection factors, including hBD-2, hBD-3 and hBD-4, are integral to the realisation of the innate protective function.

A variable expression of hBD-2 has been found in the epithelium of various organs. Importantly, hBD-2 acts synergistically with other antimicrobial protective proteins such as lysozyme and lactoferrin (Gosselink et al., 2010), and can act as a signalling molecule for monocytes, macrophages, neutrophils and immature dendritic cells (Jarczak et al., 2013). hBD-3 is thought to have bactericidal and antiviral functions (Winter and Wenghoefer, 2012). hBD-3 is released continuously at the "basal" level under relative normal conditions, and hBD-3 also acts as a signalling molecule for immune cell haemotaxis (Haarmann et al., 2015). In turn, hBD-4 has strong antimicrobial properties, as well as higher functional activity compared to hBD-2 and hBD-3 (Harder et al., 2001).

Unfortunately, antimicrobial protection factors in lung tissue in COPD have been studied poorly. Both hBD-2 and hBD-3 participate in the protection system of the native host against possible bacterial colonisation that is characteristic of COPD (Winter and Wenghoefer, 2012; Haarmann et al., 2015). However, hBD-4 release is induced by separate signalling pathways that are not similar to hBD-2 and hBD-3 activation signalling pathways (Diamond et al., 2000; García et al., 2001a, 2001b). This may partly explain why the findings of hBD-4 were significantly different from the findings of hBD-2 and hBD-3, namely reduced in COPD patients compared to control patients. Thus, the role and functions of hBD-4 in the overall environment of all factors and intercellular communication in both healthy lung tissue and COPD-affected tissue are thought to be insignificant. Respectively, elevated levels of hBD-2 and hBD-3, but not hBD-4, may indicate an association with COPD morphopathogenesis.

Lung tissue ontogenesis

In addition, this study identified the presence of various tissue factors in the tissues of relatively healthy and COPD-affected lungs in terms of

ontogenesis, indicating age changes. This part of the study significantly complements the results of previous research in morphological sciences, fundamental and clinical pulmonology.

Lung growth in a **healthy individual** occurs until the age of 10–12, while lung maturation continues until the age of 25, reaching a functional maximum at about 20 years of age. After the age of 25, lung function gradually regresses (Brandsma et al., 2017). An age-related inflammatory condition that is not associated with inflammatory diseases is called “inflammageing”. Impaired or even loss of immune function, imbalance of inflammatory and anti-inflammatory cytokines, as well as low-grade basal inflammation with predominance of inflammatory cytokines and low anti-inflammatory cytokine activity are observed in this phenomenon (Franceschi et al., 2007). In normal lung ageing, increased neutrophil counts are found in tissues, as well as increased reactive oxygen and nitrogen free radicals, gene damage, and accumulation of incompletely modified proteins, which together initiate the release of inflammatory cytokines in lung tissue (Lalley, 2013).

In the tissues of control individuals aged 9–49 years, the number of TNF-α, IL-6 and hBD-4 immunoreactive cells in various localisations increased with age. In contrast, in control individuals aged ≥ 50 years, the number of IL-4 and IL-6 immunoreactive cells in the tissue material increased at different locations with age. It is thought that an even increase in the amount of these cytokines could be associated with ageing, including progressively in the process of age-related inflammation. Changes in ageing in healthy lungs are associated with an increased findings of inflammatory cytokines IL-1α, TNF-α, IL-4, IL-6, IL-8, IL-12 and decreased finding of the anti-inflammatory cytokine IL-10 (Sharma et al., 2009; Lee et al., 2012; Lowery et al., 2013; Ito and Mercado, 2014), which all are associated with regression and inflammation of lung function.

In general, the number of IL-4 immunoreactive cells in the bronchial epithelium and blood vessels, as well as the number of hBD-4 immunoreactive cells in the bronchial cartilage increased in the tissue material of all control patients; however, the number of hBD-4 immunoreactive cells in the bronchial epithelium and connective tissue, as well as IL-6 immunoreactive alveolar macrophage numbers decreased with ageing of the individual. Studying the age-related inflammatory phenomenon, it was found that peripheral blood IL-6 levels increased with age, but it is significant that the maximum increase in IL-6 was observed in control individuals aged 50–61 years (Milan-Mattos et al., 2019); that is, it may be proposed that an increase of this and possibly other cytokines due to ageing changes is not linearly related to age, but may indicate an age range when the peak of the particular factor is observed, meanwhile, having a relatively smaller amounts of factor earlier and later than during this age range.

In control individuals aged 9–49 years, the number of IL-8, IL-7, IL-10, IL-12, Hsp-70, hBD-2, hBD-3, hBD-4 immunoreactive cells decreased in different localities with ageing. In contrast, in individuals aged ≥ 50 years, the number of IL-1α, TNF-α, IL-12, TGF-β1, Hsp-70, TIMP-2, hBD-2, hBD-4 immunoreactive cells decreased in different localisations with ageing. There are multiple studies that support ageing-related decreased production of IL-1α (Kohut and Senchina, 2004), TNF-α, IL-6 (van Duin et al., 2007; Panda et al., 2010), IL-8 (Mocchegiani et al., 2009), and IL-12 (Toapanta and Ross, 2009) by innate and acquired immunity relevant immune cells that are thought to be consequences of "immunosenescence" and "cell senescence" phenomena (Shaw et al., 2010; Bektas et al., 2017)).

However, these findings are related to general ageing-induced dysregulation of the immune system and altered ability to respond to various irritants, rather than exclusively to the development of inflammation in absolutely all cases. Since IL-7 (Gao et al., 2015), IL-10 (Nadigel et al., 2013), TGF-β1 (Rohani and Parks, 2015), TIMP-2 (Eickelberg et al., 1999), Hsp-70

(Tsan and Gao, 2004) and defensins (Yang et al., 2002) are overall associated with a protective effect; the decrease in the number of immunoreactive cells positive for these factors in terms of age could be explained by the reduced ability to perform protective functions. Since there was found a decrease in hBD-2, but an increase in the numbers of IL-4, IL-6, and TNF-α immunoreactive cells, this finding is thought to be explained by the interaction of these factors and a subsequent decrease in the amount of defensins due to ageing in relatively healthy individuals (Castañeda-Delgado et al., 2017).

Functional parameters (FEV_1 (%), FEV_1 (L) and FEV_1/FVC) in **elderly COPD patients** having ≥ 75 years of age were mostly worse compared to younger COPD individuals. The age of COPD patients was also statistically significantly associated with bronchoscopically determined chronic bronchitis, as well as epithelial metaplasia and granulation tissue findings in the histological sections. COPD in elderly individuals is known to be associated with an increased risk of complications, exacerbations, and mortality (Cortopassi et al., 2017). Quite all functional parameters in COPD patients decrease with age (Lange et al., 2015).

In COPD general pathomorphological events, surprisingly similar age-related symptoms are observed, such as cell ageing, stem cell depletion, increased oxidative stress, changes in the extracellular matrix, and a decrease in endogenous ageing molecules and protective pathways such as autophagy (Mercado et al., 2015). Therefore, COPD is often compared to the accelerated course of ageing changes. It should also be noted that ageing processes, as in relatively healthy individuals, are accelerated in the lung tissue of COPD patients, and also worsen the course of COPD (MacNee, 2009, 2016). Significantly, chronic COPD inflammation contributes to lung ageing and cell depletion (Freund et al., 2010).

In addition to the decline in lung function and structural changes caused by relatively normal ageing, COPD causes airway obstruction, remodelling

changes, inflammation, fibrosis, and mucus overproduction. Increased compensatory response of innate immunity, exaggerated chemotaxis, and accumulation of immune cells are locally associated with the development of inflammatory response conditions in the lungs of elderly COPD individuals. Impaired phagocytosis is also observed in macrophage function. Neutrophil counts in the lungs of older COPD individuals increase, promoting exaggerated protease production, lung parenchymal destruction and disease progression, as well as prolonging the inflammatory process, and generally initiating adverse tissue changes (Brandsma et al., 2017; Cho and Stout-Delgado, 2020).

The morphopathogenic course of lung ageing in COPD patients is very similar to that seen in stable COPD, such as increased findings of IL-1α, IL-6, IL-8, TNF-α and proteases (Brandsma et al., 2017; Divo et al., 2018; Zuo et al., 2019; Cho and Stout-Delgado, 2020). Such changes were also detected in the present study. In all COPD patients, the number of IL-6 immunoreactive cells in the mucosal connective tissue was higher with increasing age. In COPD patients aged 50–64 years, the number of IL-6, TIMP-2, hBD-4 immunoreactive cells was higher in different localities with increasing age, but in the age group of 65–74 – the number of IL-4 immunoreactive cells. In COPD patients aged 65–74 years and ≥ 75 years, the number of IL-1α, IL-4, IL-6, IL-12, hBD-4 immunoreactive cells at different localities was higher with increasing age, but in the age group of ≥ 75 years – IL-4, MMP-2, IL-6, IL-10. One of the mechanisms of ageing is related to the functions of IL-1α, which induces inflammatory cytokine release and total cytokine imbalance. (Freund et al., 2010). Importantly, in conditions of chronic inflammation, an increased amount of IL-10 is often found. Cytokine IL-10 in the general context with inflammatory mediators, however, reduces the course of chronic inflammation (Franceschi et al., 2007). These findings could explain the age-related increased amount of cytokines IL-1α, IL-4, IL-6, IL-12, and IL-10 detected in the studied COPD patients.

An increased predominance of TIMPs promotes accumulation of structural components of the ECM and formation of fibrosis, whereas a reduced amount of TIMPs enhances proteolysis of the ECM by a predominance of MMPs (Arpino et al., 2015). MMP-2 expression increases in the lungs with ageing and causes age-related phenotypic changes in lung fibroblasts, as well as significantly interferes with ECM regeneration (Brew and Nagase, 2010). Local MMP-2 expression causes connective tissue restructuring; however, in the elderly, MMPs are also associated with impaired innate immunity (Hansen et al. 2015). An increase in MMP-2, but a decrease in TIMP-2 and TGF-β1 in terms of ageing that was found in the study, could be associated with age-related remodelling changes. Due to the decrease of TIMP-2, lung tissue degradation changes are found in the elderly. Therefore, an increase in MMP-2 but a decrease in TIMP-2 and TGF-β1, which was found as ageing changes, could indicate more pronounced age-related ECM degradation and tissue damage.

Overall, more pronounced findings of IL-1α, IL-4, IL-6, IL-12, IL-10, MMP-2 and hBD-4 is thought to indicate the importance of these factors in the ageing process in COPD.

The numbers of IL-7 immunoreactive cells in the blood vessels of all COPD patients was decreased with ageing. In COPD patients aged 50–64 years and 65–74 years, the numbers of TNF-α, IL-7, IL-8, Hsp-70, hBD-2, hBD-3 immunoreactive cells was decreased in different localities with ageing; moreover, in oldest COPD patients aged ≥ 75 years – the numbers of IL-7 and TGF-β1 immunoreactive cells.

Contrary to what was described in the literature, decreased numbers of IL-8 immunoreactive cells were determined with increasing age of individuals. Decreased IL-8 production may be related to the impaired capacity of bronchial epithelial cells and their potential to respond to environmental irritation (Nadigel et al., 2013). This is a truly surprising phenomenon, as COPD is classically associated with elevated levels of inflammatory cytokines (such as IL-8). In

detailed study of decreased IL-8 with ageing, the authors found that IL-8 levels in COPD patients were still increased compared to "basal" IL-8 levels in healthy lung tissue (de Boer et al., 2000). Examining this assumption, the same finding was identified in the current study. Accordingly, these findings in COPD affected lung tissue should be considered as more pronounced when compared to control group tissues, but reduced in general view (absolutely) as decreased secretion of particular factor may be associated with age-related cell dysfunction. The same finding was associated with lower numbers of TNF-α, IL-7, TGF-β1, hBD-2, and hBD-3 immunoreactive cells at different localities with increasing age.

Secondly, because a close relationship between IL-10 and IL-8 was determined, it is possible that more pronounced IL-10 expression had an effect on IL-8 secretion. Also, decreased levels of TGF-β1 are associated with ageing of immune cells, as well as impaired immune function of TGF-β1 having dominant background of inflammatory cytokines (Hansen et al., 2015). In addition, despite the potentially beneficial effects of TGF-β1, accelerated cell ageing and dysfunction are also observed in COPD (Chilosi et al., 2013). Thirdly, reduced levels of IL-8 and TNF-α may be associated with prolonged inflammatory processes.

As in the control group, the reduced amount of defensins in COPD patients due to ageing could be explained by weakened protective functions and the ability of the immune system to resist the pathogens of respiratory diseases (Castañeda-Delgado et al., 2017), although the numbers of immunoreactive cells positive for these factors were higher in COPD patients than in the control group. In addition, a reduction in hBD-2 in COPD patients has been observed in viral and bacterial infections (Arnason et al., 2017; Zielinski et al., 2018) that are known as significant risk factors for worsened course of COPD, including during ageing (Zielinski et al., 2018; Cho and Stout-Delgado, 2020). As hBD-4 levels are known to be increased in acute rather than chronic airway infections (Yanagi et al., 2005), it is thought that the increase in hBD-4 with age could be explained

by greater airway reactivity to pathogens under reduced protective functions. In contrast, the numbers of Hsp-70 immunoreactive cells in COPD patients were already lower than in the control group, so the deficiency of this factor in elderly COPD patients may be associated with significant cell depletion in chronic inflammation (Wu et al., 2013).

In general, an age-related increase in the number of IL-1α, IL-4, IL-6, IL-10, IL-12, MMP-2, hBD-4 immunoreactive cells was observed, but decrease of IL-7, IL-8, TNF-α , TIMP-2, TGF-β1, Hsp-70, hBD-2, hBD-3 immuno-reactive cells in COPD patients, as well as chronic bronchitis, epithelial metaplasia, granulation tissue finding, and worse functional status in elderly COPD patients.

Thus, the most important findings of the current study in the lung tissues of relatively healthy individuals and COPD patients should be highlighted. In normal lung tissue, findings of various factors having the "baseline level" under relative health conditions was demonstrated, while maintaining tissue reactivity and the ability to prevent cell and tissue damage in response to various irritants, as well as managing lung immunity. It is important that these phenomena are found in different parts of the lungs – in the bronchial wall and alveoli, as well as in different histological structures. In COPD patients, increased numbers of IL-1α, IL-4, partly IL-6, partly IL-8, TNF-α, IL-7, IL-10, partly IL-12, TGF-β1, MMP-2, TIMP-2, hBD-2, partially hBD-3 immunoreactive cells were detected at different bronchial wall localisations, but reduced numbers of Hsp-70 and partially hBD-4 immunoreactive cells compared to the control group. These findings justify the significance of various factors related to the initiation, maintenance and regulation of the inflammatory processes linked together with remodelling events and antimicrobial defense, as well as highlight the role of impaired protective function in COPD morphopathogenesis.

4 CONCLUSIONS

1. Lung tissue in COPD patients is characterised by variable and localised non-specific changes as chronic inflammation and tissue remodelling with inflammatory cell infiltration, granulation tissue, thickened basement membrane and vascular wall, bronchial gland and smooth muscle hypeplasia and hypertrophy, as well as fibrosis and epithelial metaplasia.
2. Control lung tissue is characterised by overall low numbers of cells immunoreactive for IL-1α, IL-4, IL-6, IL-8, TNF-α, IL-7, IL-12, IL-10, TGF-β1, MMP-2 , TIMP-2, Hsp-70, hBD-2, hBD-3, and hBD-4 at different localisations. This indicates stable, continuous, and adaptive "baseline" level of these tissue factors at a relative norm. More pronounced findings of these factors in the bronchial and alveolar epithelium, and among alveolar macrophages are associated with the maintenance of local lung homeostasis, including immunity and tissue reactivity; however, in hyaline cartilage, plasticity and the potential compensatory reaction with the tissue increased.
3. COPD affected lung tissue is characterised by more pronounced findings of the inflammatory cytokines IL-1α, IL-4, IL-8 and TNF-α. An increased number of IL-10 immunoreactive cells indicates persistent anti-inflammatory response. Expressed IL-7 findings suggest activated immunomodulation. Interaction of IL-7 with cytokines IL-1α, IL-4, IL-6, IL-8, IL-10, remodelling factors TGF-β1, MMP-2, TIMP-2, cell damage marker Hsp-70, and defensins hBD-2, hBD-3, hBD-4 indicate the role of combined cytokine complex in the regulation of inflammatory and anti-inflammatory processes along with remodelling, oxidative stress and antimicrobial protection.
4. Significantly higher numbers of TGF-β1, MMP-2, TIMP-2 immunoreactive cells in COPD affected lung tissue compared to the control group indicate the role of these factors in tissue remodelling processes. Small predominance of TIMP-2 may limit tissue destruction processes, but does not individually affect

the development of fibrosis and, consequently, airway obstruction in COPD. Close relationship of these factors with cytokines IL-1α, IL-4, IL-6, IL-8, IL-12, IL-10, cell damage marker Hsp-70, and defensins hBD-2, hBD-3, hBD-4 links inflammation with remodelling. The association of TGF-β1 with remodelling and inflammatory processes justifies the dual function of this factor. Markedly low Hsp-70-containing cell numbers indicate cell depletion.

5. Pronounced findings of hBD-2 and hBD-3 (but not hBD-4) promotes antimicrobial lung protection in COPD patients, indicating an increase in total and selective antibacterial protection.
6. Distinct findings of tissue factors in bronchial epithelium, connective tissue and blood vessels prove the involvement and importance of these structures in the morphopathogenesis of COPD. In general, COPD morphopathogenesis is characterised by increased IL-1α, IL-4, IL-6, IL-8, TNF-α, IL-7, IL-10, IL-12, TGF-β1, MMP-2, TIMP-2, hBD -2, hBD-3; however, reduced Hsp-70 and hBD-4 expression that indicates high activity of persistent complex cytokine network, tissue remodelling and overall tissue antimicrobial protection.
7. In COPD, bronchoscopically determined hypetrophy and chronic bronchitis are associated with the presence of IL-8, MMP-2, hBD-3 and hBD-4 in COPD-affected lung. Epithelial metaplasia and fibrosis in the histological sections are associated with worse functional parameters and IL-6, IL-8, MMP-2. Moreover, the finding of granulation tissue is associated with a longer history of smoking and more pronounced tissue damage.
8. Ageing changes in the lungs at relative health are individual, even different for individuals in various age groups, also, are mostly characterised by a lack of inflammation and variability, even a decrease, in the number of immune cells. In turn, in the case of COPD, ageing is associated with worsening of the course of the disease, as well as persistence of inflammatory cytokines and altered remodelling with otherwise typical COPD events.

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