VILNIUS UNIVERSITY

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THE EFFECTS OF BRONCHIAL INFLAMMATION ON LUNG FUNCTION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Summary of doctoral dissertation

Biomedical sciences, Medicine (06B)

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VILNIAUS UNIVERSITETAS

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BRONCHŲ UŽDEGIMO POVEIKIS PLAUČIŲ FUNKCIJAI SERGANT LĖTINE OBSTRUKCINE PLAUČIŲ LIGA

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ABBREVIATIONS

Abbreviation	Explanation
CD4+	lymhocytes helpers
CD4+CD25+	T regulatory lymphocytes
CD8+	citotoxic lymphocytes
COPD	chronic obstructive pulmonary disease
CRP	C reactive protein
CT	computed tomography
*D*Lco	diffusing lung capacity
ESR	erythrocyte sedimentation rate
FEV_1	forced expiratory volume in 1 sec
FIV_1	forced inspiratory volume in 1 sec
FVC	forced vital capacity
GOLD	global initiative of chronic obstructive pulmonary disease
IC	inspiratory capacity
IFN-γ	interferon gama
IL	interleukin
NBT	neutrophil nitroblue tetrazolium test
NFA	neutrophil phagocytosis activity test
NK	natural killers
PCO_2	partial pressure of carbon dioxide
PO_2	partial pressure of oxygen
RV	residual volume
SaO_2	arterial oxygen saturation
TGF-β	transforming grow factor
TLC	total lung capacity
TNF-α	tumor necrosis factor alpha
VC	vital capacity

INTRODUCTION

Relevance of the study

Chronic obstructive pulmonary disease (COPD), a common preventable and treatable disease, is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients.

The exact prevalence of COPD is unknown; however, it is supposed that approximately 200 million patients (4–6% of male and 1-3% female population) are suffering from this disease worldwide. According to the World Health Organization, COPD is the 4th most common cause of death in the world: approximately 3 million of patients die from COPD annually. The mortality and morbidity COPD rates are increasing every year.

Chronic obstructive pulmonary disease is progressing slowly and constantly. The progress of the disease causes worsening of pulmonary function and symptoms of the disease, as well as decrease of quality of life. This confusing disease is often being diagnosed too late, as the beginning of the disease is almost indistinguishable.

The causes of the development, progress and persistence of airway inflammation are not exactly clear, yet. However, it is obvious that primary mechanism includes oxidative stress caused by tobacco fumes or other external factors, accumulation of inflammatory cells (foremost macrophages, neutrophils and CD8 T lymphocytes) in airway tissue, pulmonary parenchyma and activation of these cells. The inflammation encompasses large and small airways, pulmonary parenchyma and lung vessels. Proteolytic enzymes produced by macrophages and neutrophils impair the balance of proteases and anti - proteases, resulting in excess of proteolytic enzymes in the lungs. These enzymes destroy pulmonary tissue and promote the development of emphysema.

Currently, it is known that COPD stimulates systemic inflammation *per se*. The pulmonary function in COPD patients with higher markers of inflammatory activity (e.g. blood fibrinogen etc.) is markedly lower. Furthermore, in the patients with more rapid worsening of markers of inflammatory activity the pulmonary function deteriorates more rapidly, too. The exacerbation of the disease enhances inflammation of the airways and, as well, systemic inflammation. Therefore, it is supposed that more rapid decrease of

pulmonary function in patients who experience exacerbations more frequently is determined by increasing inflammation of the airways. Inflammatory proteins and other substances enter systemic blood flow and impair functions of other organs; therefore, COPD causes lesions of other organs, also. Systemic changes of whole organism are characteristic for this disease, including weight loss, dysfunction of skeleton muscles, higher risk of cardiovascular events, depression, erythraemia (occasionally – anaemia), osteoporosis, pulmonary hypertension and chronic pulmonary heart disease. These changes develop because of immobility caused by dyspnoea, systemic inflammation, hypoxia of the tissues and oxidative stress.

As with majority of chronic diseases, the phases of remission and exacerbation are characteristic for COPD. Every exacerbation, especially in event of belated diagnosis and inappropriate treatment, worsens patient's condition for a long period of time and increases the risk of death significantly. Therefore, the determination of the origin of an exacerbation is of utmost importance. An infection of respiratory airways is the most common factor stimulating the development of exacerbation. On the other hand, some of exacerbations are of non-infectious (i.e. immune) origin. It is not easy to differentiate the origin of the exacerbation, as invasive examinations (e.g. bronchoscopy or microbiology examination of bronchial aspirate) are not always available. Well known markers of activity are used in clinical practice to diagnose the character of COPD exacerbation, including white blood cell count, level of C reactive protein, red blood cell sedimentation rate, fibrinogen level, etc. Currently, attempts to find out the markers helpful for differentiation of the character of COPD exacerbation (infectious vs. non-infectious) are being made. As potential non-invasive markers of bacterial COPD exacerbation, the blood cytokines are supposed to be promising. However, the data regarding the diagnostic value of laboratory markers of COPD exacerbation are insufficient, as the data presented by various authors are contradictory.

Since the end of 2011, in clinical practice the stage of COPD was determined only taking into account FEV_1, the indicator of respiratory function. Of late years, this method raised discontent among both scientists and clinical practicians increasingly, as it did not reflect the variety of pulmonary changes characteristic of COPD and correlated with clinical symptoms and functional conditions of the patients poorly. Although the decreased values of FEV_1 and FEV_1/FVC are helpful in evaluation and classification of obstruction

of the airways, the decrease of these values is not sufficient when identifying the morphologic cause of obstruction and creating more detailed picture of the disease. The decrease of the expired air flow (obstruction) in patients suffering from COPD can be determined by various factors, including lesions of small airways, emphysema, bronchiectasis, fibrosis or combination of these factors. In order to treat COPD better, it is very important to evaluate both functional and structure changes of the airways and lungs thoroughly. Complex evaluation of these changes enables us to characterize the phenotype of the disease particular patient is suffering from and to select an optimal treatment. It is not always possible to perform a lot of comprehensive examinations in clinical practice; therefore, it may be very important to know the relationship between the indicators of respiratory function and radiologic changes of the lungs, expecting that the results of one examination (e.g., evaluation of pulmonary function) will be helpful to understand the existing changes of pulmonary structure found, for example, on computed tomography imaging.

Chronic airway inflammation in COPD is a pivotal factor determining all pathologic and functional changes of bronchi and pulmonary parenchyma that, in turn, determine systemic inflammation and hypoxemia. Although the inflammation is one of the most important factors of this disease, the exact place of anti-inflammatory drugs (inhalation glucocorticosteroids) in the algorithm of COPD treatment is not clearly established. These drugs alleviate the course of the disease and slow down the deterioration of pulmonary function, improve quality of life in some patients; however, positive effect of the drugs is absent or even negative in other patients, as the medications increase the risk of infectious complications (pneumonias) and death. As the results of recent studies are inconsistent and contradictory, a hypothesis is created, stating that autoimmune response causing permanent inflammation is being initiated after primary harmful stimulus (e.g. tobacco smoking) takes place in some patients suffering from COPD, e.g. in patients suffering from chronic bronchitis, chronic inflammation persists and determines progression of the disease resulting in COPD. Data allowing to confirm or to reject this hypothesis are insufficient, yet. This study was started while expecting to find out the relationship between inflammation of the bronchi and respiratory function during various clinical phases of COPD and to define this relationship better.

The aim of the study

To investigate relationship between inflammation of the airways (bronchi) and pulmonary function.

Objectives

1. To examine the levels of peripheral blood cytokines of patients suffering from COPD during exacerbation and remission, in order to detect non-invasive marker/markers of COPD that will enable us to differentiate infectious origin of exacerbation from non-infectious.
2. To investigate relationship between radiology changes of the lungs and respiratory function in patients suffering from chronic obstructive pulmonary disease.
3. To find out whether it is possible to evaluate changes of airway inflammation using classical inflammation and bacterial markers in patients suffering from COPD. To evaluate dependence of these markers on COPD clinical phase.
4. To evaluate the count of regulatory T lymphocytes (CD4+CD25+), as cells, possibly decreasing inflammation, in blood of the patients suffering from COPD and compare the results with results obtained from the group of patients who are not suffering from COPD.
5. To examine the amount of cells with CD25+ marker in mucosa of bronchi of patients suffering from COPD and compare this result with the result obtained in the control groups of non-smokers and smokers who are not suffering from COPD.

Scientific novelty

In practical work, the problem of timely diagnosis of exacerbation of the disease remains still important, despite significant achievements in research and treatment of COPD. The search of potential non-invasive markers enabling to diagnose the exacerbations of COPD timely and to determine the origin of exacerbation (bacterial vs. immune) is ongoing. From the point of possible markers of COPD, cytokines are promising. Although the blood levels of IL-10 and TNF-α were examined in patients suffering from COPD in several studies, the results of these studies were contradictory. Unlike other researchers, we tried to detect relationship between exacerbation of the disease, bacterial

colonisation of the airways and blood cytokines levels; therefore, we had compared the patients suffering from COPD both during remission and exacerbation. For this purpose, we used a novel method of investigation and examined intracellular cytokines instead of extracellular ones.

We found no publications concerning application of NBT and NFA examinations for diagnosis of COPD exacerbations or origin of these exacerbations, nevertheless these examination were available in clinical practice. We were the first researchers who used these tests for examination of the patients suffering from COPD.

In accordance with the hypothesis stating that relative deficiency of regulatory T cells causing uncontrolled autoimmune inflammation in organisms of some smokers may be one of the causes of development of COPD, we have examined the amount of regulatory T cells in the blood and bronchial mucosa of the patients suffering from COPD. The results of our study had confirmed this hypothesis.

Principal statements of defence

1. The exacerbation of COPD is reflected by changes of blood cytokines levels, i.e. increased level of IL-10 and decreased level of TNF-α.
2. The staging of patients suffering from COPD based on FEV_1 only, does not reveal entire functional and structure pulmonary changes suitably.
3. The levels of blood markers of inflammation activity depend on the clinical phase of COPD. The increase of airway inflammation is properly reflected by blood inflammatory markers activity, including CRP, ESR, fibrinogen, WBC count, as well as by results of microbiology examination of bronchial aspirate. In COPD, the diagnostic value of NBT, NFA and procalcitonin tests is not significant.
4. The dysfunction of immune system of the body, i.e. deficiency of CD4+CD25+ (regulatory T lymphocytes) resulting in insufficient suppression of airway inflammation (possibly autoimmune), is important for development of COPD. The most marked deficiency of these cells is characteristic of patients suffering from severe and very severe COPD.

MATERIALS AND METHODS

Patient selection

The regional Ethics Committee approved this prospective study protocol.

The material studied was collected since February, 2009 till April, 2012 at the Centre of Pulmonology and Allergology of Vilnius University Hospital Santariškių Clinics.

Criteria for inclusion into the study:

- Male and female patients elder than 18 years old, suffering from chronic obstructive pulmonary disease (all stages) and relatively healthy subjects (controls).
- The subjects who understand the essence of the study, who had signed Subject's Informed Consent Form.

Exclusion criteria:

- Concomitant disease that may contort the results of the study, including systemic disease of connective tissue, systemic vasculitis, other known autoimmune disease, former or present tumour of any organ, exacerbation of any concomitant disease (e.g. kidneys, liver) that may distort the results of the study.
- Bronchial asthma
- Allergic rhinitis
- Pneumonia
- Alcoholism, drug addiction
- Vulnerable subjects (subordinated persons, persons suffering from mental disability)
- Use of systemic glucocorticosteroids, cytostatics.

A total of 99 subjects were enrolled into the study, including 73 patients suffering from COPD (all stages) and 26 healthy persons (controls). All 99 subjects underwent comprehensive examination of lung function (spirometry with bronchodilation test, plethysmography, and gas diffusion examination). Blood samples to examine activity of inflammation (WBC count, CRP, ESR, fibrinogen, NFA and procalcitonin) were obtained from 32 patients. Blood NBT test was performed for 73 COPD patients and 26 control subjects, also. All 99 study subjects underwent bronchoscopy; for 43

patients and 26 control subjects the biopsy of bronchial mucosa was performed, all these patients provided blood samples for examination of CD4+CD25+ (Treg) cells. The bronchoscopy revealed endobronchial tumor and confirmed the diagnosis of lung cancer in one patient suffering from COPD. This patient was directed for surgery and discontinued participation in our study.

Computed tomography imaging was performed for 38 patients. The samples for examination of blood cytokines were obtained from 24 subjects suffering from COPD.

Examination of blood markers of inflammation activity

The following blood indicators were examined during exacerbation and remission:

- Leukocyte count, erythrocyte sedimentation rate (ESR), haemoglobin level – were examined using *Sysmex XE-5000 and Coulter LH-780* analysers;
- The blood level of C reactive protein (CRP) was measured using automatic analyser *Architect ci-8200* and reagents made by *Abbott*, USA;
- The blood plasma level of fibrinogen was measured using *Stago Compact* analyser and reagents made by *Stago*, Germany;
- The blood plasma level of procalcitonin were measured using *Advia Centaur CP* analyser and reagents made by *Siemens*, Germany;
- The neutrophil nitroblue tetrazolium (NBT) test: the method was designed to evaluate the function of neutrophils using nitroblue tetrazolium; this substance undergoes reduction under the influence of neutrophil oxygen metabolism and forms black precipitate in cytoplasm. The test was evaluated using light microscopy.
- Neutrophil phagocytic activity (NFA) – neutrophils were incubated with polystyrene latex particles; phagocytosis was evaluated using light microscopy.

Examination of blood CD4+CD25+ lymphocytes

Monoclonal Becton Dickinson Simultest^{TM} antibodies (CD45/CD14, CD3/CD8, CD4/CD25, CD8/CD25) marked with two colour fluorochromes - fluorescein isothiocyanate (FITC) and phycoerhythrin (PE) were used for examination. Simultest LeucoGATE (CD45/CD14) monoclonal antibody was used to detect the region of lymphocyte analysis and Simultest Control γ1/γ2a (IgG1 FITC/IgG2a PE) monoclonal antibody was used to detect margin between positively and negatively coloured cells and to reject possible non-specific scintillation. The samples were analysed using FACSCalibur cytometer (Becton Dickinson).

Examination of blood cytokines

In order to evaluate peripheral blood levels of substances with short half-life, Th1 and Th2 cell cytokines – interleukins (IL) IL-2, IL-4, IL-6, IL-10, tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), we inserted the test-tube with blood into a container with ice immediately. At the laboratory the serum was separated using centrifuge and frozen at - 80°C; the serum was stored till examination, then. CBA human Th1/Th2 Cytokine Kit II, Becton Dickinson, was used for examination of cytokines. Several analytes of the same sample were simultaneously evaluated quantitatively. Activated microspheres (~650 nm/FL3) of different fluorescence intensity coated with specific monoclonal antibodies were used for examination of each cytokine. Corresponding standards of solutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 1:256) and negative control were prepared before every examination.

10 μl of monoclonal antibody of cytokine examined were added into test-tube with serum of each patient and staining with detection reagent was performed, then. Washing with buffer solution from the kit was performed after incubations. The calibration balls for calibration of flow cytometer were prepared before each analysis of cytokines. On the same day, the samples were analysed using flow cytometer FACS Calibur (*Becton Dickinson*, USA) and FCAP Array ^{TM} software.

Computed tomography imaging of the chest

Computed tomography (CT) imaging was performed using 64 section computed tomography equipment (GE Healthcare, Milwaukee, Wisconsin, USA). After maximal inspiration and holding the breath for 10 s., the scan from clavicles to diaphragm was performed in caudal-cranial direction using spiral 1.25 mm sections. CT small dose protocol was used for this investigation (tube voltage < 120 kVp and current strength of the tube < 80 mA). The changes of structure, including presence of emphysema, bullae, bronchiectasis and pulmonary fibrosis were evaluated. The changes were quantitatively assessed according the number of pulmonary lobes damaged.

Honeycomb picture of pulmonary tissue, consisting of groups of 3 – 10 mm large cysts, was chosen as a criterion of pulmonary fibrosis (Fig. 1). The deformations of bronchial and vascular pattern, interlobular lines and traction bronchiectasis were considered to be the signs of pulmonary fibrosis, also. Emphysema was diagnosed when areas of pulmonary tissue with decreased density and unclear margins were present (Fig. 2). A rounded $\leq$ 10 mm large air density structure with thin walls was considered to be a bullae. A bronchus with diameter larger than that of accompanying artery (e.g. "signet ring" sign in cross-section; Fig. 4), insufficient narrowing of the bronchus peripherally and clearly visible bronchial structure less than 10 mm from pleura were considered to be a bronchiectasis. CT images were independently assessed by two blinded radiologists. In event the assessments were different (11 % of cases), the final decision was made taking into account the lesser damage.

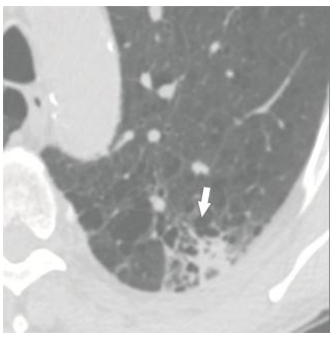

Figure 1. Pulmonary fibrosis. Sub-pleural cysts (3 – 10 mm in diameter), stripes of pulmonary tissue and groups of unevenly enlarged airways with clear margins are present ("net" and "honeycomb" type picture of pulmonary tissue).

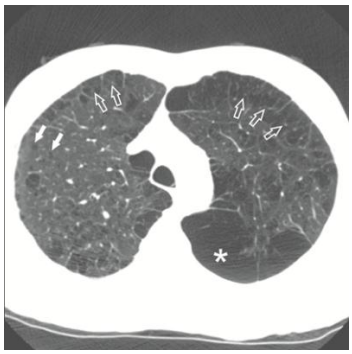

Figure 2.. Emphysema. Signs of central lobular (indicated by white arrows) and paraseptal (indicated by hollow arrows) emphysema. Bullae (marked with the asterisk) in superior lobe of the left lung is present.

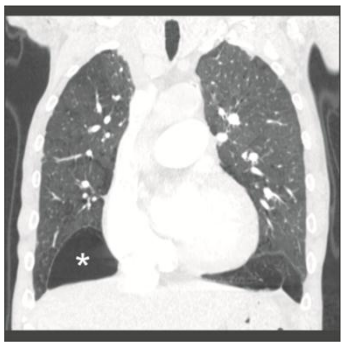

Figure 3. Bullae. The structure with thin walls and air density, larger than 10 mm is present (marked with the asterisk).

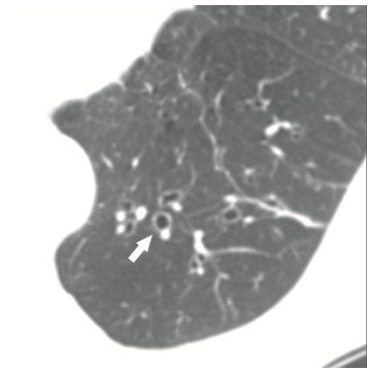

Figure 4. Bronchiectasis. The diameter of bronchi is larger than that of accompanying artery ("signet ring" sign).

Examinations of lung function

All the patients underwent spirometry, measurement of lung volume (using the whole body plethysmography) and evaluation of diffusing capacity (by means of one inspiration method). The examinations of respiratory function were performed using

Vmax Encore (*Viasys® Healthcare*, USA) equipment. The data of examinations were assessed in accordance with criteria of Consensus of European Respiratory Society/American Thoracic Society (ERS/ATS) (2005). Obstruction was diagnosed when FEV_1/FVC ratio was lower than lower limit of normal. The grade of obstruction severity was evaluated according to FEV_1 value. The hyperinflation of the lungs was diagnosed when increased total lung capacity (TLC) and air trapping – increased residual volume (RV) were present. The impairment of gas diffusion was reflected by carbon monoxide transfer factor (*D*Lco) value lower than lower limit of normal.

Bronchoscopy

Bronchoscopy procedures were performed using fiber bronchoscopes manufactured by Olympus and Pentax (Japan). The procedure was performed when the patient was sitting. The patients received 1.0 ml of atropine sulphate 0.1 % subcutaneously for premedication. Anaesthesia of nose and throat was performed using Lidocaine 10 % spray solution

administered into both nostrils and throat. Trachea and bronchi were anaesthetized using 20 ml of Lidocaine 2 % solution. During the bronchoscopy, bronchial secretions were evacuated and a sample for microbiology culture was taken; using the tweezers, a sample of mucosa for biopsy (consisting of 6 pieces) was taken from the ridges of sub-segmental bronchi of the inferior lobe of the right lung.

Examination of CD25+ cells of bronchial mucosa

Histology tissues fixed using 10 % formalin buffer solution and impregnated with paraffin were examined. Three micron thin slices were used for histochemistry and immuno-histochemistry (IHC) examinations. The micro-preparations stained with haematoxylin and eosin were scanned using magnification of 20X by means of Aperio *Scan Scope GL* scanner (*Aperio Technologies, Vista, CA,* USA). The pathologist marked the sites of the tissue examined in scanned picture of whole tissue section.

The expression of protein markers in paraffin sections, examined in current study was analysed using IHC method by means of *Ventana BenchMark XT* automatic staining equipment (*Ventana Medical Systems, Tucson,* Arizona,USA). After removal of paraffin

using xylene and dehydratation by means of ethyl alcohol, the paraffin sections were inserted into *Ventana* washing solution. *Ventana* cell conditioning solution was used to restore the epitopes of antigens (pH 8,5) 100^{o}C 36 min. Later on, the sections were incubated with monoclonal antibodies 37°C 16 min., using *Ventana Ultraview DAB* detection system. At the end of IHC reaction, the sections were contrasted by means of *Mayer's* haematoxylin and covered with covering glasses. Whole section specimen of breast tumour were used for positive IHC test control; the negative control was performed in the same sections while omitting the stage of primary placing of the antibody used in IHC reaction. The digital images were scanned by means of *Aperio ScanScope GL* scanner of objective glasses (*Aperio Technologies, Vista, CA*, USA) with magnification 20X.

Statistical analysis

Statistical calculations were performed using SPSS 17.0 statistical package. At first, the difference between the groups was assessed using χ^2 test for assessment of normality of the values. In event the values were found to be distributed not in accordance with the normal law, Mann-Whitney –U non- parametric test was used. The difference between the groups was also evaluated using Student's criterion t-test. Pearson's correlation quotient (r) was used to assess correlation, also. The strength of correlation was evaluated in accordance with the scale of correlations quotient value. The data were considered to be statistically reliable when $p < 0.05$. The data were presented as expression of the mean and standard deviation.

RESULTS

Blood cytokines

The level of blood cytokines was examined in 24 patients (Tab. 1, Fig. 5). The level of the majority of cytokines showed no difference while comparing remission and exacerbation phases (Tab. 5).We have found out that in comparison with remission, the

level peripheral blood IL-10 is statistically reliably higher in patients who suffer from exacerbation.

Bronchial aspirates of the majority of the patients in exacerbation group (71.4 %) revealed growth of pathogenic bacteria (*Streptococcus pneumoniae* 35.3%, *Moraxella catharhalis* 23.6 %, *Haemophillus influenzae* 17.6 %, *Klebsiella* 11.7 %, *Pseudomonas aeruginosa* 5.9 % etc.). So, the exacerbation of COPD in majority of the patients studied by us was of bacterial origin. The positive bacteriologic culture in remission group was present in 20 % of the patients, only. We performed further distribution of exacerbation and remission groups, in accordance with the results of aspirate culture; the patients were distributed into sub-groups of bacterial, non-bacterial exacerbation and remission. It was found out, that in the sub-group of non-bacterial exacerbation the level of TNF-α was statistically reliably higher (4.67±2.38 pg/ml), in comparison with the sub-group of bacterial exacerbation (1.88±2.49 pg/ml; p = 0.03).

Table 1. Demographic data of study subjects

Variable	**Remission (n=10)**	**Exacerbation (n=14)**	**p value**
Gender (m/f)	10/0	13/1	0.06
Age (years)	61±16	68±6	0.13
Smoking history (pack years)	16±9	35±14	0.002
FEV_1 (% pv)	47±12	41±12	0.009
White blood cells ($x10^9$ l)	7.6±0.9	13.2±2.3	0.002
CRP (mg/dl)	7±6	43±22	0.007
Fibrinogen (g/l)	4±0.8	5±0.6	0.006
Bacteria isolated (number of study subjects)	2	10	0.004

Note: the data are presented as the mean and standard deviation, unless otherwise indicated; m – males; f – females; pack year - number of years when the patient smokes one pack (20 cigarettes) per day; pv – predicted volume; FEV_1 – volume that has been exhaled at the end of the first second of forced expiration; CRP – C reactive protein.

After distribution of the patients independently of the exacerbation or remission phase into two groups according to bacterial growth in bronchial aspirate (i.e. those who had

bacterial growth and those who did not) the higher level of IL-10 was found in patients who had positive bacterial culture (11.66±6.27 pg/ml vs. 8.32±1.29 pg/ml); however, this difference statistically was not reliable. On the other hand, in the group of the patients who showed no bacterial growth of bronchial aspirate, the blood level of TNF-α was reliably higher in comparison with that in patients who had positive bacterial cultures of bronchial aspirate (5.18±2.58 pg/ml, *vs* 2.4±1.14 pg/ml; $p< 0.01$).

We found out no statistically reliable correlation between blood cytokines and other blood indicators of inflammation.

Table 2. Level of blood cytokines during remission and exacerbation of COPD.

Cytokines	**Remission (n=10)**	**Exacerbation (n=14)**	**p value**
IL–2 (pg/ml)	12.5±3.9	9.1±5.3	0.101
IL–4(pg/ml)	4.2±1.1	3.7±1.6	0.319
IL–6 (pg/ml)	6.6±1.8	8.2±5.9	0.48
IL–10(pg/ml)	6.9±1.6	20,0±9.3	0.024
TNF–α (pg/ml)	3.8±1.9	2.4±2.1	0.08
IFN–γ, (pg/ml)	3.1±0.2	2.8±1.0	0.304

Note: the data are presented as mean and standard deviation.

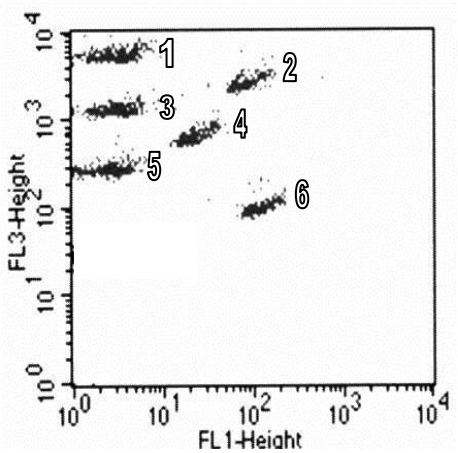

Figure 5. Cloudlets of cytokines in scatter diagram. 1 – IL–2, 2 – IL–4, 3 – IL–6, 4 – IL–10, 5 – TNF– α, 6 – INF– γ.

Relationship between radiology and pulmonary function changes

This study included 38 subjects suffering from COPD; in accordance with severity of the disease, the patients were distributed into 2 groups (the group I included patients with severe and very severe COPD and the group II consisted of the patients suffering from moderate and mild COPD). Demographic and respiratory function data are presented in Tables 3 and 4.

Table 3. Demographic data of study subjects.

Variable	**Total**	**COPD severe and very severe**	**COPD mild and moderate**
Number of study subjects	38	18	20
Males/females	34/4	15/3	19/1
Age (years)	66.9±11.5	65.4±12.0	68.4±11.3
Smoking history (pack years)	34.1±15.5	32.1±16.9	36.0±14.4
Duration of the disease (years)	12.1±5.2	13.9±7.5	10.0±4.1

Note: Pack year - number of years when the patient smokes one pack (20 cigarettes) per day. The data are presented as mean and standard deviation.

Table 4. Respiratory function data of study subjects

Variable	**Total** (n=38)	**COPD severe and very severe** (n=18)	**COPD mild and moderate** (n=20)
FEV_1, % pv.	50.4±13.9	39.3±7.9*	60.3±10.0*
FVC, % pv.	90.2±21.6	82.1±22.2*	97.4±18.6*
FEV_1/FVC, %	44.2±9.7	39.1±8.6*	48.7±8.3*
TLC, % pv	118.7±24.2	123.2±29.3	114.8±18.9
VC, % pv	90.6±20.2	84.9±21.6	95.3±18.2
RV, % pv	174.3±61.1	193.9±77.2	157.5±37.6
*D*Lco, % pv	65.2±21.4	52.8±19.9*	74.5±17.7*
pO_2 (mm Hg)	57.6±11.6	55.7±14.6	60.7±6.4
pCO_2 (mm Hg)	39.1±5.9	40.9±7.0	37.1±4.2
sO_2, %	89.5±7.0	87.4±9.2	92.1±2.3

Note:* $p<0.05$ after comparing group I and II; pv – predicted volume, mm Hg – millimetres of mercury. The data are presented as mean and standard deviation.

There were no statistically reliable differences in CTI changes between the groups of mild and severe bronchial obstruction (according to GOLD) stage (Table 5).

Table 5. Changes of pulmonary structure of study subjects.

Variable	**Total** (n=38)	**COPD severe and very severe** (n=18)	**COPD mild and moderate** (n=20)
Emphysema	4.2±1.5	3.9±1.7	4.6±1.2
Bullae	0.9±1.3	0.7±1.4	1.1±1.2
Fibrosis	3.3±1.3	3.3±1.4	3.2±1.3
Bronchiectasis	3.0±1.7	3.2±1.6	2.7±1.8

Note: The data are presented as a number of pulmonary lobes. There was no statistically reliable difference between the groups. The data are presented as mean and standard deviation.

During remission, there were no statistically significant differences between the groups in pulmonary volumes, values of blood gases and gas diffusion, also (Figures 6 and 7). However, the values of TLC, RV, and capillary pCO_2 in patients with more severe stage of bronchial obstruction suffering from COPD exacerbation were markedly higher and *D*Lco, pO_2, sO_2 were markedly lower, in comparison with these of patients with less severe stage ($p<0.05$) (Figures 8 and 9).

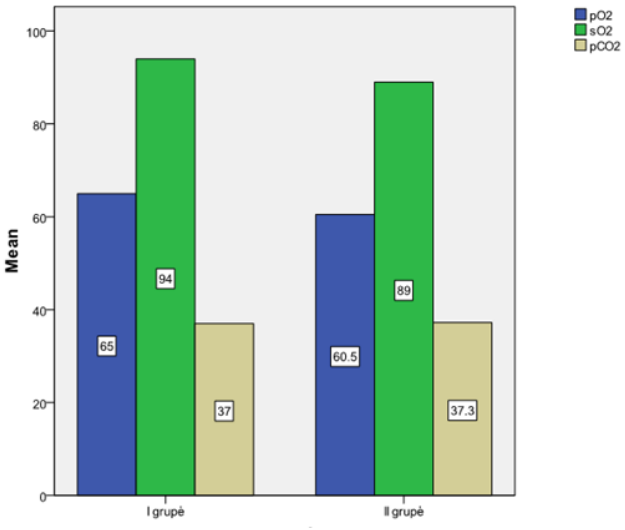

Figure 6. Values of blood gases during remission. The values between the groups of patients with more severe and less severe stages of bronchial obstruction did not differ significantly.

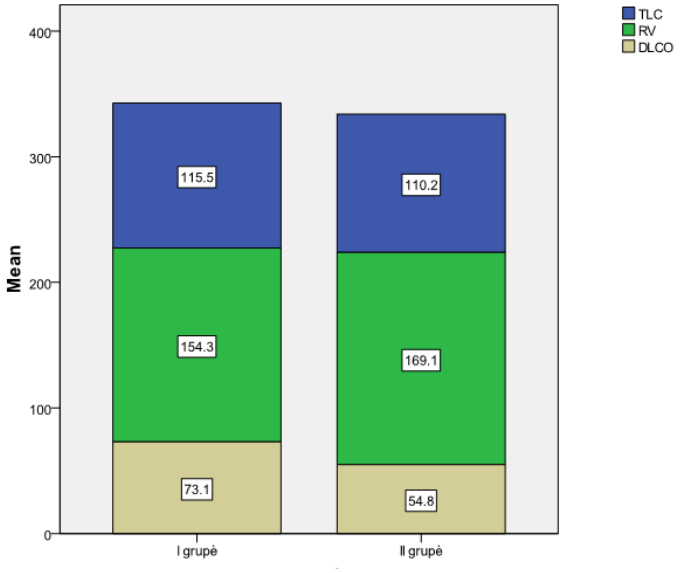

Figure 7. Pulmonary volumes and diffusing capacity during remission. The values of pulmonary volumes and gas diffusing capacity through alveolar capillary membrane did not significantly differ in patients with more and less severe stage of bronchial obstruction.

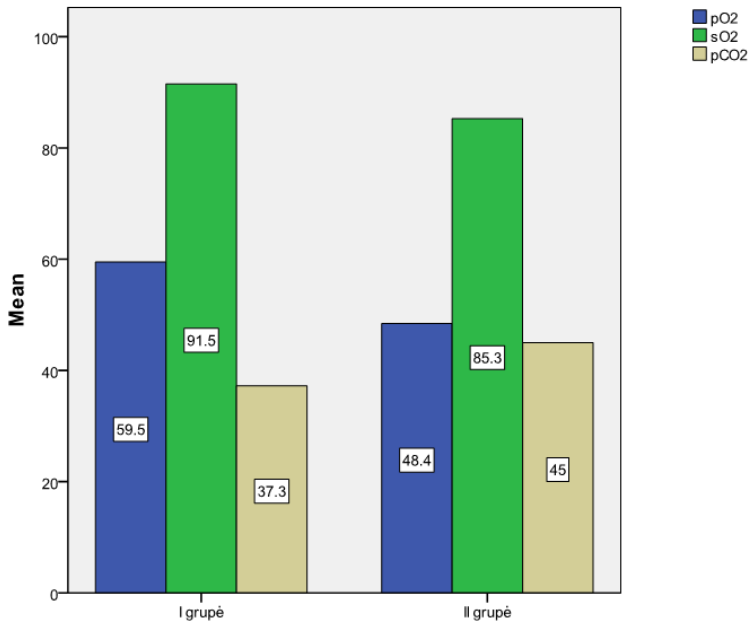

Figure 8. Values of blood gases during exacerbation. During exacerbation, the values of oxygenation were significantly worse and pCO2 was higher in patients with more severe stage of bronchial obstruction, in comparison with these of patients with less severe stage of bronchial obstruction.

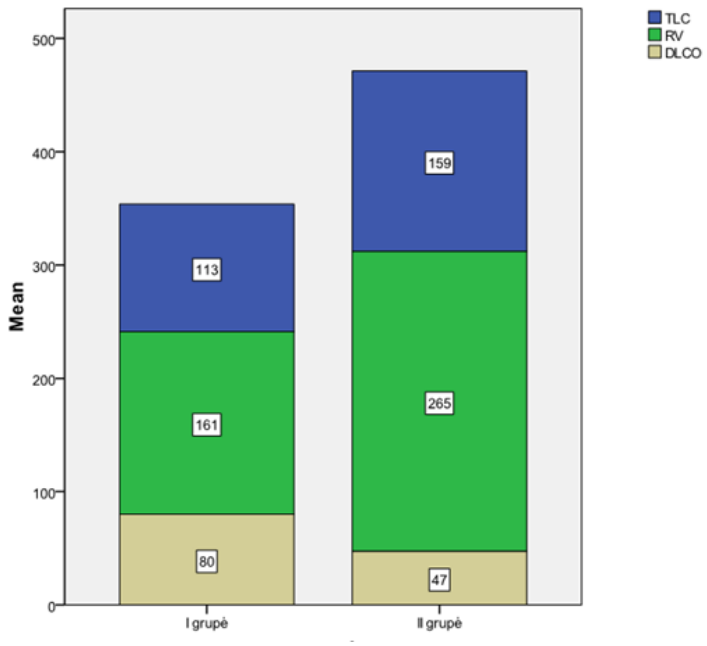

Figure 9. Pulmonary volumes and diffusing capacity during exacerbation. In the group of patients with more severe stage of bronchial obstruction, markedly higher residual pulmonary volume, lung inflation (i.e. air trapping) and lower gas diffusing capacity were observed during exacerbation of the disease, in comparison with patients suffering from less severe stages of bronchial obstruction.

We have found out linear correlations between bronchiectasis, emphysema and the rate of exacerbations (r=0.4; p=0.014; Fig.10), between pulmonary fibrosis and presence of bronchiectasis (r=0.4; p=0.014); the value of *D*Lco correlated negatively with the extent of pulmonary fibrosis and the rate of exacerbations of the disease (r = – 0.4; p=0.02; Fig. 11). We have also found negative correlation between TLC, RV and pO_2, sO_2 (r = –0.4, p=0.009) and positive correlation between TLC, RV and pCO_2 (r =0.4, p = 0.027).

Blood markers of inflammation activity during remission and exacerbation of COPD

The study included a total of 73 patients (Tab. 6). The majority of the patients in exacerbation group revealed positive pathogenic bacteria culture of bronchial aspirate, including *Streptococcus pneumoniae* (35.3 %), *Moraxella catharhalis* (23.6 %), *Haemophillus influenzae* (17.6 %), *Klebsiella* (11.7 %), *Staphylococcus aureus* (5 %),

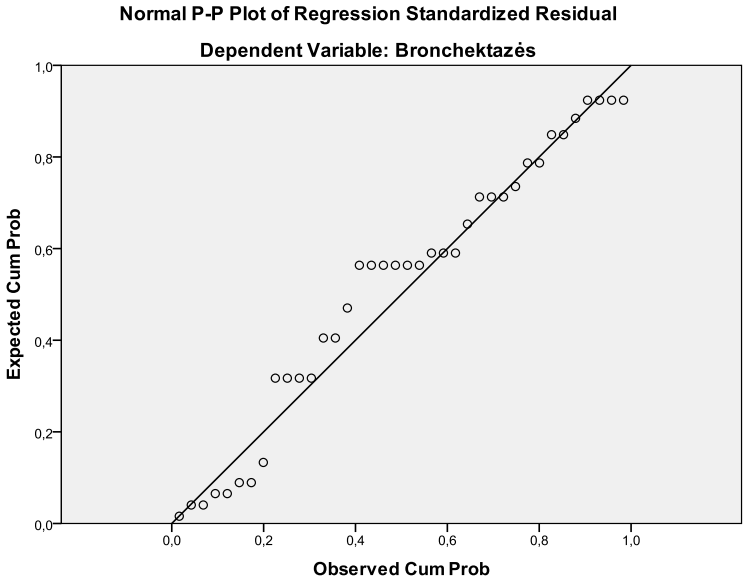

Fig. 10. Linear correlation between bronchiectasis and the rate of COPD exacerbations.

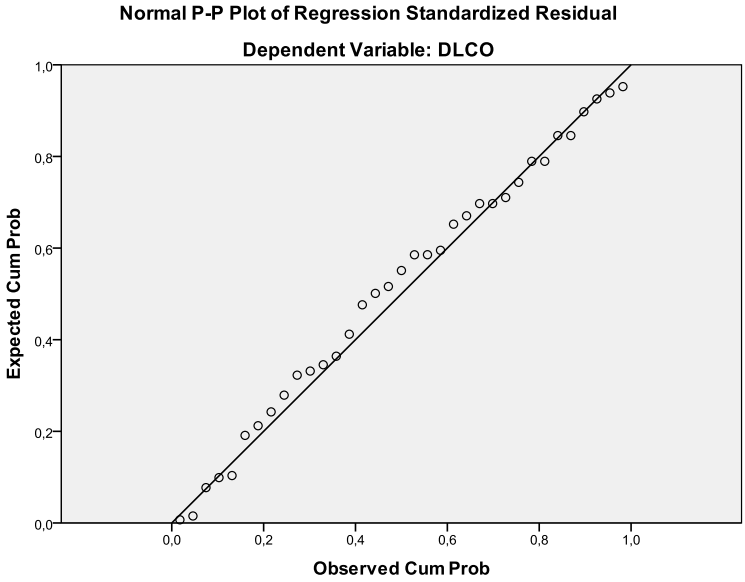

Fig 11. Linear correlation between gas diffusion and pulmonary fibrosis.

Pseudomonas aeruginosa (5.9 %); note: more than one infection causing bacteria was found in 20 % of the patients. So, the majority of our patients had COPD exacerbations of bacterial origin. On the other hand, 20 % of the patients in remission group had positive bacterial cultures, also. It was found out that the levels of CRP, fibrinogen, WBC and ESR values in the blood of patients suffering from COPD differed markedly during remission and exacerbation (Tab. 7).

Table 6. Demographic data of study subjects.

Variable	**Remission (n=55)**	**Exacerbation (n=18)**	**p value**
Gender (m/f)	52/3	16/2	0.37
Age (years)	62.9±13.6	67.4±12.5	0.22
History of smoking (in pack years)	30.9±16.4	33.05±14.7	0.62
FEV1 (% pv)	52.9±16.4	44.7±16.7	0.08

Note: the data are presented as the mean and standard deviation, unless otherwise indicated; m – males; f – females; pack year - number of years when the patient smokes one pack (20 cigarettes) per day; pv – predicted volume; FEV_1 – volume that has been exhaled at the end of the first second of forced expiration; CRP – C reactive protein.

We found out no increase in blood procalcitonin levels in patients suffering from COPD exacerbation. There was no significant increase in either NBT, or in NFA, during COPD exacerbation. There were no differences of NBT and NFA between patients with COPD and healthy ones; however, there was a good correlation between these values themselves. The direct correlations between CRP and fibrinogen, another protein of acute inflammation phase ($r = 0.8$, $p<0.01$), ESR ($r = 0.6$ $p< 0.01$), WBC count ($r = 0.7$, $p< 0.01$), presence of bacteria in bronchial aspirate ($r = 0.6$; $p<0,01$) and, as expected, direct correlations between fibrinogen and ESR ($r = 0.5$; $p<0,05$), WBC count ($r = 0.4$; $p<0.05$ presence of bacteria in bronchial aspirate ($r = 0.4$; $p<0.05$) were found out.

Blood T regulatory lymphocytes (CD4+CD25+)

CD4+CD25+ blood cells were examined for 69 study subjects, including 43 patients suffering from COPD and 26 healthy persons. The patients suffering from COPD were distributed into two groups, in accordance with severity of the disease: a group (I) of

Table 7. Blood markers of inflammation activity during COPD remission and exacerbation

Variable	Remission	Exacerbation	p value
CRP (mg/l) (n = 32)	1.95±1.4	36.0±42.5	0.01
Fibrinogen (g/l) (n = 32)	3.5±0.45	4.8±1.35	0.005
WBC count (10^9/l) (n = 32)	7.0±1.65	11.2±3.9	0.002
ESR (mm/h) (n = 32)	20.6±10.8	48.4±32.9	0.04
NBT (%) (n = 73)	35.7±17.5	36.2±17.7	0.92
NFA (%) (n = 32)	34.9±19.6	25.2±23.9	0.25

Note: the data are presented as the mean and standard deviation, unless otherwise indicated; CRP – C reactive protein; NBT – nitroblue tetrazolium test; NFA – neutrophil phagocytic activity.

patients with mild and moderate disease and group (II) of patients suffering from severe and very severe COPD. The control group consisted of smokers and non-smokers. The results of the study are presented in Tables 8 – 10.

Table 8. CD4+CD25+ and T regulatory cell count in all groups

Examined group	CD4+CD25+*	CD4+CD25+^{bright}(Treg)
COPD(GOLD I–II) n=22	575±330	59±32
COPD (GOLD III–IV) n=21	376±235	47±26
Controls (smokers) n=14	610±217	75±27
Controls (non-smokers) n=12	392±157	59±29

Note: the count of cells is presented in absolute numbers per ml^3. The data are presented as the mean and standard deviation

The comparison of COPD and control groups demonstrated no statistically significant difference either in total number of CD4+CD25+ cells, or in CD4+CD25+^{bright}(Treg). However, in the group of patients suffering from severe and very severe COPD, the count of these cells was found to be significantly lower, in comparison with healthy smokers (Table 9).

The difference between smoking and non-smoking controls was found to be statistically significant, also: the count of CD4+CD25+ lymphocytes was markedly higher in healthy smokers than in non-smokers (Tab. 10).

No significant correlation between respiratory function and CD4+CD25+ blood cells were found.

Table 9. Comparison of CD4+CD25+ and CD4+CD25+ ^{bright} blood cells in the groups of severe , very severe COPD and controls.

Type of cells	**COPD (GOLD III-IV) (n=20)**	**Control (smokers) (n=14)**	**p value**
CD4+CD25+	376±235	610±217	0.01
CD4+CD25+^{bright}	47±26	75±27	0.03

Note: the count of cells is presented in absolute numbers per ml^3. The data are presented as mean and standard deviation.

Table 10. Comparison CD4+CD25+ and CD4+CD25+^{bright} blood cells between both control groups.

Type of cells	**Controls (non-smokers) (n=11)**	**Controls (smokers) (n=14)**	**p value**
CD4+CD25+	392±157	610±217	0.02
CD4+CD25+^{bright}	42±19	59±29	0.007

Note: the count of cells is presented in absolute numbers per ml^3. The data are presented as the mean and standard deviation.

Evaluation of CD25+ lymphocytes of bronchial mucosa

The biopsies of bronchial mucosa were examined for the same 69 subjects who underwent assessment of CD25+ blood cell count. The same groups of study subjects were compared, including the group of patients with severe and very severe COPD, mild and moderate COPD, healthy smokers and non-smokers (Tab. 11). A reliable difference between COPD and controls was found out: in the group of COPD patients the count of CD25+ cells was markedly lower (Tab. 12, 13).

Table 11. Data of examination of CD25+ cells of bronchial mucosa biopsy

Examined group	**CD25+**
COPD (GOLD I-II) n=22	112±53
COPD (GOLD III-IV) n=21	88±39
Controls (smokers) n=14	130±62
Controls (non-smokers) n=12	132±57

Note: the count of CD25+ cells is presented in absolute numbers per mm^2. The data are presented as the mean and standard deviation.

Table 12. CD25+ cell count in biopsies of bronchial mucosa in COPD and control groups.

Cell type	COPD (n=43)	Controls (n=26)	p value
CD25+	100.9±50.4	131.4±64.1	0.04

Note: the count of CD25+ cells is presented in absolute numbers per mm^2. The data are presented as the mean and standard deviation.

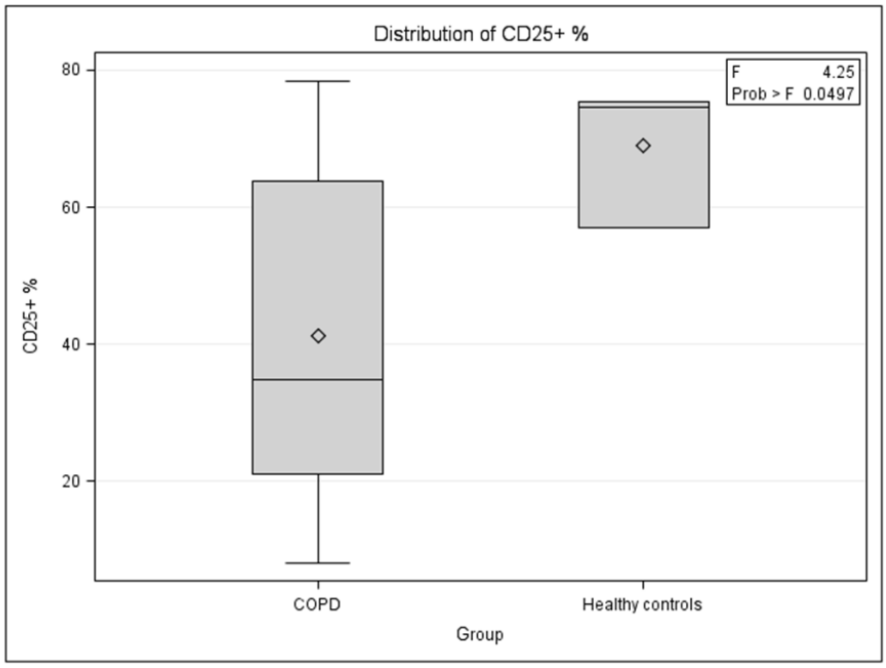

Table 13. Box-and whisker diagram represents significantly lower bronchial mucosa CD25+ cell count in COPD group, in comparison with control group.

CONCLUSIONS

1. The levels of blood markers of inflammation activity depend on clinical phase of COPD. During exacerbation, the intensification of inflammation of the airways is well reflected by classic blood markers of inflammatory activity, including CRP, ESR, fibrinogen, white blood cell count and, as well, the results of bronchial aspirate examination. NBT, NFA and procalcitonin tests, from the points of view of diagnosis of inflammation of the airways and treatment of exacerbation, are not important in patients suffering from COPD.

2. The changes of the levels of blood cytokines, including increased IL-10 and decreased TNF-α level, reflect an exacerbation of COPD. It is very possible that

these cytokines may be valuable laboratory markers of COPD clinical phase, enabling one to differentiate bacterial exacerbation of COPD from non-bacterial.

3. The classification of the patients suffering from COPD into stages taking into account only FEV_1 value reveals the entirety of structure and functional changes of the lungs insufficiently. The relationship between radiology and functional changes of the lungs is not unambiguous. The worsening of gas diffusion index reflects the development of pulmonary fibrosis. The more frequent exacerbations of COPD are related to more severe changes of pulmonary structure (i.e., bronchiectasis and emphysema). The patients with poorer functional condition during remission (III and IV groups of severity of bronchial obstruction according to GOLD) experience markedly greater impairment of functional condition of respiratory system during exacerbation, in comparison with that of the patients in mild to moderate severity stages of COPD.

4. The dysfunction of immune system of the body plays the role in development of COPD, also, as the inflammation of the airways (possibly autoimmune) may be supressed inadequately due to insufficiency of CD4+CD25+ (T regulatory) lymphocytes.

RECOMMENDATIONS FOR PRACTICE

1. It is recommended to examine white blood cell count, CRP, ESR, fibrinogen levels in event of worsening of condition of the patient suffering from COPD, as the increase of the levels of these markers demonstrate exacerbation of COPD reliably.
2. In unclear cases it is useful to perform the examinations of blood cytokines IL-10 and TNF–α, in order to differentiate the exacerbation of COPD from remission, because the increase of IL-10 and decrease of TNF–α levels are characteristic of exacerbation.

3. However a comprehensive examination of pulmonary function partially reflects changes of the structure of the lung, the information obtained is not sufficient enough to evaluate these changes appropriately; therefore, it is recommended to perform (at least once) computed tomography of the chest for the patients suffering from COPD. This examination is very useful for evaluation of severity of impairment of pulmonary parenchyma, assessment of phenotype of the disease and selection of an optimal treatment.

LIST OF PUBLICATIONS

1. Šileikienė V, Nargėla R, Danila E. The significancy of lung function evaluation in COPD. Health Sciences 2009; 1(61): 2201-2205.

2. Šileikienė V, Nargėla R, Danila E. Peculiarities of airway inflammation in chronic obstructive pulmonary disease. Paediatric pulmonology and allergology 2009; 12(1): 4162-4170.

3. Šileikienė V, Danila E. Phenotypes of chronic obstructive pulmonary disease: case reports. Theory and Practice in Medicine 2010; 16(3): 297-302.

4. Šileikienė V, Jurgauskienė L, Malickaitė R, Danila E. Blood cytokines concentration reliance on clinical phase of chronic obstructive pulmonary disease. Laboratory Medicine 2011; 13(49): 26-30.

5. Šileikienė V, Zeleckienė I, Norkūnienė J, Matačiūnas M, Danila E. The relationship between radiologic findings and lung function impairment in chronic obstructive pulmonary disease. Paediatric pulmonology and allergology 2012; 15(1): 4932-4940.

6. Šileikienė V, Norkūnienė J, Bagdonaitė L, Danila E. Variations of blood inflammatory biomarkers in chronic obstructive pulmonary disease. Laboratory medicine 2012; 14: 2(54): 75-79.

7. Šileikienė V, Zeleckienė I, Matačiūnas M, Norkūnienė J, Danila E. The relationship between radiologic findings and lung function impairment in chronic obstructive pulmonary disease. Eur Respir J 2012; 40 (Suppl. 56): 414s.

PRESENTATIONS

1. Šileikienė V. Clinical exercise testing significancy in COPD. The annual conference of Lithuanian Society of pulmonologists, 2010 06 04.

2. Šileikienė V, Jurgauskienė L, Malickaitė R, Danila E. Blood cytokines concentration reliance on clinical phase of chronic obstructive pulmonary disease. Conference „Pulmonology, allergology and clinical immunology – 2011“, Kaunas. 2011 04 27.

3. Šileikienė V, Zeleckienė I, Matačiūnas M, Norkūnienė J, Danila E. The relationship between radiologic findings and lung function impairment in chronic obstructive pulmonary disease. European respiratory society annual congress, Viena 2012 09 03 (P2299).

BRIEF INFORMATION ABOUT THE AUTHOR

Virginija Šileikienė was born on April 4, 1971 in Vilnius, Lithuania. After finishing Vilnius Žirmūnai high school she studied at the Medical faculty of Vilnius University. She graduate Vilnius University in 2000 and obtained the diploma of medical doctor. She started her residence of pulmonology at the Medical faculty of Vilnius University after one-year internship. Virginija Šileikienė obtained the license of pulmonologist in 2005 and started to work at Vilnius University hospital Santariškių clinics as a pulmonologist. From 2008 until 2012 she maintained the doctor’s thesis at the Vilnius University Faculty of Medicine. The main field of her scientific interest is pulmonary function testing, chronic obstructive pulmonary disease, lung cancer.

Since 2005 Virginija Šileikienė is an active member of Lithuanian Society of Pulmonologists. She is a member of European Respiratory Society. More than ten articles of V. Šileikienė with co-authors were published in reviewed and International scientific journals.

REZIUMĖ

Santrumpos

Santrumpa	Paaiškinimas
CD4+	limfocitai helperiai
CD4+CD25+	T reguliaciniai limfocitai
CD8+	citotoksiniai limfocitai
LOPL	lėtinė obstrukcinė plaučių liga
CRB	C reaktyvusis baltymas
KT	kompiuterinė tomografija
*D*Lco	difuzinė plaučių geba
ENG	eritrocitų nusėdimo greitis
FEV_1	angl. *forced expiratory volume in 1 sec* - forsuoto iškvėpimo tūris per pirmąją sekundę
FVC	angl. *forced vital capacity* - forsuota gyvybinė talpa
GOLD	globalinė lėtinės obstrukcinės plaučių ligos iniciatyva
IFN γ	gama interferonas
IL	interleukinas
NMT	neutrofilų nitromėlio tetrazolio testas
NFA	neutrofilų fagocitozės aktyvumo testas
PCO_2	dalinis anglies dioksido slėgis
PO_2	dalinis deguonies slėgis
RV	liekamasis tūris
SaO_2	deguonies saturacija
TLC	angl. *total lung capacity* – bendroji plaučių talpa
TNF α	navikų nekrozės faktorius alfa
VC	angl. *vital capacity* – gyvybinė talpa

Įvadas

Tikslus sergamumas lėtine obstrukcine plaučių liga (LOPL) nežinomas, tačiau manoma, kad pasaulyje šia liga serga apie 200 milijonų žmonių (4–6 proc. vyrų ir 1–3 proc. moterų). Pasaulinės sveikatos organizacijos duomenimis, šiuo metu LOPL pasaulyje užima 4-ą vietą tarp dažniausių mirties priežasčių. Kasmet pasaulyje nuo LOPL miršta apie 3 milijonus žmonių. Sergamumas ir mirtingumas nuo LOPL kasmet didėja.

Lėtinė obstrukcinė plaučių liga nuolatos pamažu progresuoja. Tai lemia plaučių funkcijos blogėjimą, ligos simptomų stiprėjimą ir gyvenimo kokybės blogėjimą. Ši klastinga liga dažnai diagnozuojama per vėlai, nes jos pradžia beveik nepastebima.

Kodėl atsiranda, sustiprėja ir persistuoja kvėpavimo takų uždegimas iki šiol tiksliai nežinoma. Tačiau akivaizdu, kad pirminis mechanizmas yra tabako dūmų ir (ar) kitų išorinių veiksnių sukeltas oksidacinis stresas, uždegimo ląstelių, visų pirma makrofagų, neutrofilų ir CD8 T limfocitų priplūdimas į kvėpavimo takus bei plaučių parenchimą ir šių ląstelių aktyvinimas. Uždegimas apima stambiuosius ir smulkiuosius kvėpavimo takus, plaučių parenchimą bei plaučių kraujagysles. Dėl makrofagų ir neutrofilų išskiriamų proteolizinių fermentų sutrinka proteazių ir antiproteazių pusiausvyra. Susidaro proteolizinių fermentų perteklius plaučiuose. Proteoliziniai fermentai ardo plaučių audinį, skatina atsirasti emfizemą.

Dabar jau žinoma, kad pati lėtine obstrukcine plaučių liga skatina sisteminį uždegimą. LOPL sergančių ligonių, kurių uždegimo rodikliai (fibrinogeno kiekis kraujyje ir kt.) yra didesni, plaučių funkcija yra gerokai blogesnė. Ligos paūmėjimas sustiprina kvėpavimo takų ir sisteminį uždegimą. Į sisteminę kraujotaką patekę uždegimo baltymai ir kitos medžiagos sutrikdo kitų organų funkciją, todėl LOPL pažeidžia ne tik plaučius ir kvėpavimo takus. Šiai ligai būdingi sisteminiai organizmo pokyčiai – svorio mažėjimas, griaučių raumenų disfunkcija, didesnė kardiovaskulinių įvykių rizika, depresija, eritremija, kartais anemija, osteoporozė, plautinė hipertenzija ir lėtinė plautinė širdis.

LOPL, kaip ir daugumai lėtinių ligų, būdinga remisijos ir paūmėjimo fazių kaita. Kiekvienas paūmėjimas, ypač nelaiku ir netinkamai gydomas, ilgam pablogina ligonio būklę ir labai padidina mirties riziką. Todėl ypač svarbu nustatyti paūmėjimo kilmę. Dažniausiai LOPL paūmėjimą skatina kvėpavimo takų infekcija. Tačiau dalis paūmėjimų yra neinfekcinės (imuninės) kilmės. Atskirti paūmėjimo kilmę nėra paprasta, nes ne visuomet galima atlikti invazinius tyrimus, tokius kaip fibrobronchoskopiją ir bronchų aspirato mikrobiologinį tyrimą. Klinikinėje praktikoje LOPL paūmėjimo pobūdžiui nustatyti kartu su klinikiniais simptomais naudojami gerai žinomi aktyvumo rodikliai – leukocitų skaičius kraujyje, C reaktyviojo baltymo koncentracija, eritrocitų nusėdimo greitis, fibrinogenas ir kai kurie kiti. Pastaruoju metu bandoma aptikti žymenų, kurie padėtų atskirti infekcinę paūmėjimo priežastį

nuo neinfekcinės. Daug tikimasi iš kraujo citokinų, kaip potencialių neinvazinių bakterinio LOPL paūmėjimo rodiklių. Tačiau vis dar nepakanka duomenų apie LOPL paūmėjimo laboratorinių žymenų diagnostinę vertę, nes įvairių autorių radiniai šiuo klausimu prieštaringi.

Klinikinėje praktikoje lėtine obstrukcine plaučių liga sergantys ligoniai iki 2011 metų pabaigos buvo stadijuojami tik pagal vienintelį kvėpavimo funkcijos rodiklį – FEV_1. Pastaraisiais metais toks ligonių suskirstymas kėlė vis daugiau nepasitenkinimo tiek tarp mokslininkų, tiek tarp gydytojų praktikų, nes neatspindėjo LOPL būdingos plaučių pokyčių įvairovės ir blogai koreliavo su ligonių klinikiniais simptomais ir funkcine būkle Sumažėjęs iškvepiamo oro srautas (obstrukcija) sergant LOPL gali būti sąlygotas įvairių priežasčių – smulkiųjų kvėpavimo takų pažeidimo, emfizemos, bronchektazių, fibrozės ar jų derinių Norint geriau gydyti LOPL sergančius ligonius, labai svarbu kompleksiškai įvertinti tiek funkcinius, tiek struktūrinius kvėpavimo takų ir plaučių pokyčius. Tai padeda geriau apibūdinti konkretaus paciento ligos fenotipą ir parinkti tinkamiausią gydymą

Ankstesniuose tyrimuose gautų duomenų įvairovė, neatitikimas ir prieštaringumas, iškėlė hipotezę, kad daliai LOPL sergančių ligonių po pirminio žalojančio veiksnio (pvz., tabako rūkymo) stimulo, inicijuojamas autoimuninis atsakas, lemiantis nuolatinį uždegimą. Kol kas duomenų, leidžiančių paneigti, ar patvirtinti šią hipotezę nepakanka. Šis tyrimas pradėtas tikintis, kad pavyks rasti ir geriau apibūdinti bronchų uždegimo ir kvėpavimo funkcijos sąsajas įvairių LOPL klinikinių fazių metu.

Tyrimo tikslas

Ištirti kvėpavimo takų (bronchų) uždegimo sąsajas su plaučių funkcija.

Tyrimo uždaviniai

1. Ištirti asmenų, sergančių LOPL, periferinio kraujo citokinų koncentraciją ligos paūmėjimo ir remisijos metu, tikintis rasti neinvazinį LOPL paūmėjimo žymenį (žymenis), leisiantį atskirti infekcinę paūmėjimo kilmę nuo neinfekcinės.
2. Ištirti radiologinių plaučių pokyčių ir kvėpavimo funkcijos rodiklių sąsajas sergant lėtine obstrukcine plaučių liga.

3. Ištirti, ar apie kvėpavimo takų uždegimo pokyčius sergant LOPL galima spręsti iš klasikinių uždegimo ir bakterijų žymenų pokyčių kraujyje. Įvertinti šių kraujo žymenų priklausomybę nuo LOPL klinikinės fazės.
4. Nustatyti T reguliacinių limfocitų (CD4+CD25+), kaip galimai uždegimą slopinančių ląstelių, kiekį LOPL sergančių ligonių kraujyje ir palyginti su LOPL nesergančių asmenų grupe.
5. Ištirti CD25+ žymenį turtinčių ląstelių kiekį LOPL sergančių ligonių bronchų gleivinėje ir palyginti jį su LOPL nesergančių rūkančių ir nerūkančių asmenų kontrolinėmis grupėmis.

Mokslinė darbo reikšmė ir naujumas

Nežiūrint didelių pasiekimų tiriant ir gydant LOPL, praktiniame darbe iki šiol išlieka savalaikio ligos paūmėjimo nustatymo problema. Nuolat ieškoma potencialių neinvazinių žymenų, kurie galėtų padėti laiku diagnozuoti LOPL paūmėjimą ir galimai nustatyti jo kilmę (bakterinis ar imuninis). Daug tikimasi iš citokinų, kaip potencialių LOPL paūmėjimo žymenų. Nors pavieniuose darbuose tirta IL-10 ir TNF-α koncentracija LOPL sergančių ligonių kraujyje [144,145], tačiau tyrimų duomenys prieštaringi. Ne taip kaip kiti tyrėjai, mėginome aptikti ryšį tarp ligos paūmėjimo, bakterinės kvėpavimo takų kolonizacijos ir kraujo citokinų koncentracijos, todėl palyginome LOPL sergančius ligonius paūmėjimo ir remisijos metu. Tam tikslui pirmą kartą panaudojome naują metodiką, ištirdami ne ekstraląstelinius, bet viduląstelinius citokinus.

Neradome literatūros duomenų apie NMT ir NFA tyrimų pritaikymą LOPL paūmėjimams nustatyti ar jų kilmei diferencijuoti, nors šie tyrimai yra prieinami mūsų klinikinėje praktikoje. Pirmieji panaudojome šiuos testus sergantiems LOPL ligoniams tirti.

Remdamiesi hipoteze, kad santykinis T reguliacinių ląstelių trūkumas ir dėl to vykstantis nekontroliuojamas autoimuninis uždegimas kai kurių rūkalių organizme galėtų būti viena iš LOPL atsiradimo priežasčių, ištyrėme T reguliacinių ląstelių kiekį LOPL sergančių ligonių kraujyje ir bronchų gleivinėje. Mūsų gauti rezultatai patvirtino šią hipotezę.

Ginamieji teiginiai

1. LOPL paūmėjimą atspindi kraujo citokinų koncentracijos pokyčiai – padidėjusi IL-10 koncentracija ir sumažėjusi TNF-α koncentracija.
2. Sergančiųjų LOPL suskirstymas į stadijas tik pagal FEV_1 rodiklį nepakankamai atskleidžia plaučių funkcinių ir struktūrinių pokyčių visumą.
3. Kraujo uždegimo aktyvumo žymenų koncentracija priklauso nuo LOPL klinikinės fazės. Kvėpavimo takų uždegimo sustiprėjimą LOPL paūmėjimo metu gerai atspindi klasikiniai kraujo uždegimo aktyvumo žymenys – CRB, ENG, fibrinogenas, leukocitų skaičius bei bronchų aspirato mikrobiologinio tyrimo rezultatai. NBT, NFA ir prokalcitoninas neturi diagnostinės vertės kvėpavimo takų uždegimui ir jo paūmėjimui vertinti sergant LOPL.
4. LOPL atsiradimui turi įtakos organizmo imuninės sistemos disfunkcija – CD4+CD25+ (T reguliacinių) limfocitų trūkumas ir dėl to nepakankamai slopinamas kvėpavimo takų (galimai autoimuninis) uždegimas. Didžiausia šių ląstelių stoka būdinga sergantiems sunkia ir labai sunkia LOPL.

Tyrimo medžiaga ir metodai

Vilniaus regioninis biomedicininių tyrimų etikos komitetas 2009-02-10 išdavė leidimą (Nr. 1/013) atlikti šį perspektyvinį tyrimą. Tiriamoji medžiaga rinkta nuo 2009 metų vasario iki 2012 metų balandžio mėnesio VšĮ Vilniaus universiteto ligoninės Santariškių klinikų Pulmonologijos ir alergologijos centre. Įtraukimo į tyrimą kriterijai: vyresni kaip 18 m. vyrai ir moterys, sergantys lėtine obstrukcine plaučių liga (visos stadijos) bei sąlyginai sveiki asmenys (kontrolinė grupė). Asmenys suprantantys tyrimo esmę ir pasirašę informuoto asmens sutikimo formą. Neįtraukimo į tyrimą kriterijai: gretutinė liga, galinti iškreipti tyrimo rezultatus – sisteminė jungiamojo audinio liga, sisteminis vaskulitas, kitos žinomos autoimuninės ligos, bet kurio organo buvęs ar esamas navikas, bet kurios gretutinės ligos (pvz., kepenų, inkstų), galinčios turėti įtakos tyrimo rezultatams, paūmėjimas, bronchinė astma, alerginis rinitas, pneumonija, alkoholizmas, narkomanija, pažeidžiami asmenys (pavaldūs, protinę negalią turintys asmenys), sisteminių gliukokortikosteroidų, citostatikų vartojimas. Tiriamieji tirti pakopine metodika. Visiems tiriamiesiems (n=99) išsamiai ištirta kvėpavimo funkcija (atlikta spirometrija

su bronchus plečiančiu mėginiu, pletizmografija, dujų difuzijos tyrimas). Visiems tiriamiesiems (n=99) atliktos bronchoskopijos: Iš jų – 24 sergantiems LOPL ligoniams paimta medžiaga iš bronchų mikrobiologiniam pasėliui. 69 asmenims (43 ligoniams ir 26 sveikiems kontrolinės grupės tiriamiesiems) bronchoskopijos metu paimtos bronchų gleivinės biopsijos Vienam LOPL sergančiam ligoniui atlikus bronchoskopiją buvo rastas endobronchinis navikas ir patvirtinta plaučių vėžio diagnozė. Šis pacientas nukreiptas chirurginiam gydymui ir toliau mūsų tyrime nedalyvavo. 32 ligoniams paimtas kraujas uždegimo aktyvumo rodikliams (leukocitų skaičiui, CRB, ENG, fibrinogenui, NFA ir prokalcitoninui) nustatyti. Kraujo NMT rodiklis ištirtas visiems 73 LOPL grupės ir 26 sveikiems asmenims. 43 ligoniams ir 26 kontrolinės grupės asmenims paimtas kraujo mėginys CD4+CD25+ (Treg) ląstelėms nustatyti. 24 sergantiems LOPL asmenims paimtas kraujas citokinų (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-ɣ)koncentracijai ištirti. 38 ligoniams atliktos krūtinės ląstos kompiuterinės tomografijos.

Rezultatai

Daugumos citokinų koncentracijų skirtumo paūmėjimo ir remisijos fazių metu nebuvo. Nustatėme, kad LOPL paūmėjimo metu periferiniame kraujyje rasta statistiškai patikimai didesnė IL–10 koncentracija, palyginus su remisijos grupės pacientais. Pagal aspirato iš bronchų bakteriologinio pasėlio duomenis paūmėjimo ir remisijos grupes dar suskirsčius į bakterinio ir nebakterinio paūmėjimo ir remisijos pogrupius, nebakterinio paūmėjimo pogrupyje nustatyta statistiškai patikimai didesnė TNF–į koncentracija (4,67±2,38 pg/ml), palyginus su bakterinio paūmėjimo pogrupiu (1,88±2,49 pg/ml, p = 0,03)

Visus tiriamuosius, nepriklausomai nuo paūmėjimo ar remisijos fazės, suskirsčius į dvi grupes pagal bakterijų augimą aspirate iš bronchų (į tuos, kurių bronchų aspirate bakterijos neaptiktos ir tuos, kurių aspirate bakterijos aptiktos), didesnę IL–10 koncentraciją nustatėme tos grupės ligoniams, kuriems buvo teigiamas bakteriologinis pasėlis (11,66±6,27 pg/ml, palyginti su 8,32±1,29 pg/ml), tačiau šis skirtumas nebuvo statistiškai patikimas. Tačiau grupėje, kurios ligoniams bakterijų bronchų aspirate nebuvo aptikta, TNF–α koncentracija kraujyje buvo patikimai

didesnė negu tiriamųjų, kurių bronchų aspirate bakterijų rasta (5,18±2,58 pg/ml, palyginus su 2,4±1,14 pg/ml, p< 0,01).

Radiologinių ir funkcinių plaučių sąsajų tyrime dalyvavo 38 asmenys sergantys LOPL, kurie buvo suskirtyti į 2 grupes pagal ligos sunkumą (I grupę sudarė sunkia ir labai sunkia LOPL, o II grupę – vidutinio sunkumo ir lengva LOPL sergantieji ligoniai). Statistiškai reikšmingo skirtumo pagal KT vaizduose rastus pokyčius tarp lengvesnės ir sunkesnės GOLD bronchų obstrukcijos sunkumo stadijos grupių ligonių nebuvo. Taip pat nebuvo statistiškai reikšmingo plaučių talpų, kraujo dujų bei dujų difuzijos rodiklių skirtumo tarp grupių ligos remisijos metu. Tačiau sunkesnės bronchų obstrukcijos sunkumo stadijos grupės ligonių, kuriems buvo LOPL paūmėjimas, TLC, RV, kapiliarinio kraujo pCO_2 rodikliai buvo gerokai didesni, o *D*Lco, pO_2, sO_2 – mažesni, palyginus su lengvesnės stadijos (I grupės) ligoniais (p<0,05). Nustatėme, kad bronchektazių ir emfizemos buvimas teigiamai koreliavo su paūmėjimų dažniu (r=0,4, p=0,014, o pneumofibrozė – su bronchektazių buvimu (r=0,4, p=0,014). *D*Lco rodiklio vertė neigiamai koreliavo su plaučių fibrozės apimtimi ir ligos paūmėjimų dažniu (r = – 0,4, p=0,02). Be to, nustatėme neigiamą TLC ir RV rodiklių koreliaciją su pO_2 ir sO_2 (r = –0,4, p = 0,009) bei teigiamą – su pCO_2 (r = 0,4, p = 0,027) rodikliais.

Daugumai paūmėjimo grupės ligonių (71,4 %) bronchų aspirate išaugo patogeninės bakterijos: *Streptococcus pneumoniae* 35,3 %, *Moraxella catharhalis* 23,6 %, *Haemophillus influenzae* 17,6 %, *Klebsiella* 11,7 %, *Staphylococcus aureus* ir *Pseudomonas aeruginosa* po 5 % (pastaba: 20% tiriamųjų buvo išskirtas daugiau negu vienas infekcijos sukėlėjas). Taigi, didžiajai daliai mūsų tiriamųjų LOPL paūmėjimas buvo bakterinės kilmės. Tačiau 20 % remisijos grupės pacientų taip pat išaugo bakterijos. Nustatėme, kad CRB, fibrinogeno koncentracija, leukocitų skaičius ir ENG sergančiųjų LOPL kraujyje reikšmingai skyrėsi ligos paūmėjimo ir remisijos metu. Prokalcitonino koncentracijos padidėjimo kraujyje LOPL paūmėjimo metu nenustatėme. Nei neutrofilų NMT mėginys, nei NFA rodiklis reikšmingai nepadidėjo LOPL paūmėjimo metu, tačiau viršijo normos ribas (neutrofilų NMT mėginio norminė vertė 15–25%, o NFA 20–25%). NBT ir NFA reikšmių skirtumo tarp sergančiųjų LOPL ir sveikų asmenų taip pat nebuvo, nors šie žymenys gerai koreliavo

tarpusavyje. Nustatyta tiesioginė CRB rodiklio koreliacija su kitu ūminės fazės baltymu fibrinogenu (r = 0,8, p<0,01), ENG (r = 0,6 p< 0,01), leukocitų skaičiumi (r = 0,7, p< 0,01) ir bakterijų buvimu bronchų aspirate (r = 0,6, p<0,01), kaip ir tikėtasi – tiesioginė fibrinogeno koreliacija su ENG (r = 0,5, p<0,05), leukocitų skaičiumi (r = 0,4, p<0,05) ir bakterijų buvimu bronchų aspirate (r = 0,4, p<0,05).

Palyginus LOPL ir kontrolinę grupes bendrai statistiškai reikšmingo nei bendro CD4+CD25+ ląstelių nei CD4+CD25+^{bright}(Treg) skirtumo tarp grupių nenustatyta. Tačiau sergančiųjų sunkia ir labai sunkia LOPL grupėje aptiktas reikšmingai mažesnis šių ląstelių skaičius, palyginti su rūkančiais, tačiau nesergančiais LOPL asmenimis (376±235 vs 610±217, p = 0,01).

Statistiškai patikimas skirtumas nustatytas ir tarp rūkančių bei nerūkančių kontrolinės grupės asmenų: rūkančiųjų kraujyje nustatyta gerokai daugiau CD4+CD25+ limfocitų, negu nerūkančiųjų. Ištyrus bronchų gleivinės CD25+ limfocitus rastas patikimas skirtumas tarp LOPL ir kontrolinės grupės tiriamųjų – LOPL grupėje CD25+ ląstelių buvo gerokai mažiau (100,9±50,4 vs 131,4±64,1 p = 0,04).

Išvados

1. Kraujo uždegimo aktyvumo žymenų koncentracija priklauso nuo LOPL klinikinės fazės. Kvėpavimo takų uždegimo sustiprėjimą LOPL paūmėjimo metu gerai atspindi klasikiniai kraujo uždegimo aktyvumo žymenys – CRB, ENG, fibrinogenas, leukocitų skaičius bei bronchų aspirato mikrobiologinio tyrimo rezultatai. NMT, NFA ir prokalcitoninas neturi diagnostinės vertės kvėpavimo takų uždegimui ir jo paūmėjimui vertinti sergant LOPL.
2. LOPL paūmėjimą atspindi kraujo citokinų koncentracijos pokyčiai – padidėjusi IL-10 koncentracija ir sumažėjusi TNF-α koncentracija. Labai tikėtina, kad šie citokinai galėtų būti vertingi laboratoriniai LOPL klinikinės fazės žymenys, taip pat padedantys atskirti bakterinį LOPL paūmėjimą nuo nebakterinio.
3. Sergančiųjų LOPL suskirstymas į stadijas tik pagal FEV_1 rodiklį nepakankamai atskleidžia plaučių funkcinių ir struktūrinių pokyčių visumą. Radiologinių ir plaučių funkcijos pokyčių sąsaja nėra vienareikšmė. Blogėjantis dujų difuzijos rodiklis atspindi atsiradusią plaučių fibrozę sergant LOPL. Dažnesni LOPL

paūmėjimai susiję su sunkesniais struktūriniais plaučių pokyčiais – bronchektazėmis ir emfizema. Ligoniams, kurių funkcinė būklė esant remisijai yra sunkesnė (GOLD III ir IV bronchų obstrukcijos sunkumo grupės), paūmėjimo metu kvėpavimo sistemos funkcinė būklė sutrinka gerokai labiau negu lengvesnės funkcinės stadijos ligoniams.

4. LOPL atsiradimui turi įtakos organizmo imuninės sistemos disfunkcija – CD4+CD25+ (T reguliacinių) limfocitų trūkumas ir dėl to nepakankamai slopinamas kvėpavimo takų (galimai autoimuninis) uždegimas.

Praktinės rekomendacijos

1. Pablogėjus sergančiojo LOPL paciento būklei rekomenduojama tirti kraujo leukocitų skaičių, CRB, ENG ir fibrinogeną, nes šių rodiklių koncentracijos padidėjimas patikimai rodo LOPL paūmėjimą.
2. Neaiškiais atvejais, norint atskirti LOPL paūmėjimą nuo remisijos naudinga atlikti kraujo citokinų – IL–10 ir TNF–α tyrimą, nes LOPL paūmėjimo metu padidėja IL–10 ir sumažėja TNF–α koncentracija.
3. Nors išsamus plaučių funkcijos tyrimas iš dalies atspindi struktūrinius plaučių pokyčius, gaunama informacija nėra pakankama juos tinkamai įvertinti, todėl sergantiesiems LOPL rekomenduojama nors kartą atlikti krūtinės ląstos kompiuterinę tomografiją. Šis tyrimas labai naudingas plaučių parenchimos pažeidimo sunkumui vertinti, ligos fenotipui nustatyti ir optimaliam gydymui parinkti.