

Mikroparticle and L-arginine pathway examinations in stable and exacerbated COPD patients

PhD Thesis

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Abbreviations

- α -AT: alfa-1 antitrypsin
- ADMA: asymmetric dimethylarginine
- ADP: adenosine diphosphate
- AECOPD: COPD exacerbation
- Alb: albumin
- ANOVA: variance analysis
- ASCO: American Society of Clinical Oncology
- ATS: American Thoracic Society
- BAL: bronchioalveolar lavage
- BB: beta receptor blocker
- BMI: body mass index
- BTS: British Thoracic Society
- C: Celsius degree
- CAT: COPD Assesment Test
- CBQC: COPD Biomarker Qualification Consortium
- CD: Cluster of differentiation
- CCQ: Clinical COPD Questionnaire
- COPD: Chronic Obstructive Pulmonary Disease
- COPD-AE: COPD acute exacerbation
- COX-2: ciclooxigenase-2
- CCL-18: Chemokine (C-C motif) ligand 18
- CRP: C-reactive protein
- CXCL-10: C-X-C motif chemokine 10
- CT: computed tomography
- CVD: Cardiovascular disease
- DLCO: CO-diffusions capacity
- ECLIPSE: Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points
- ELFO: electrophoresis
- eNOS: endothel orogonated nitrogen monoxide synthetase
- Eo: eosinophil leukocyte
- ERS: European Respiratory Society
- ERV: expiration reserve volume
- FDA: U.S. Food and Drug Administration
- FeNO: Exhaled air nitrogen-monoxide fraction
- FEV1: forced expiratory volume in 1 second
- FVC: forced vital capacity
- fvs: Leukocyte
- GM-CSF: granulocyte-monocyte colony stimulating factor
- GOLD: Global Initiative for Chronic Obstructive Lung Disease
- hsCRP: high sensitivity C-reactive protein
- H₂O₂: hydrogen-peroxide
- HRCT: high resolution computed tomography
- IC: inspiratory capacity
- ICAM-1: intercellular adhesion molecule-1
- ICS: inhaled corticosteroid
- IgA: immunoglobulin A
- IgG: immunoglobulin G
- IgM: immunoglobulin M
- IL: interleukin
- IL-6: interleukin 6
- IL-8: interleukin 8
- INF: interferon α

- iNOS: inducible N-monoxide syntase
- ITP: idiopatic thrombocytopenic purpura
- i.v.: intravenous
- IVC: inspiratory vital capacity
- LABA: Long Acting Beta2 Agonist
- LAMA: Long Acting Muscarinic Antagonist
- LTB-4: leukotrien-B4
- MMA: monometil arginine
- MMP: matrix metalloproteinase
- mMRC: modified Medical Research Council dyspnoe scale
- MP: microparticle
- MPlF1: myeloid progenitor inhibitory factor 1
- MPO: myeloperoxidase
- mRNS: messenger ribonuclein acid
- Neu: neutrophil leukocyte
- NÉ: non interpretable
- NO: nitrogen-monoxide
- NOS: nitrogen monoxide syntetaze
- nNOS: neuronal nitrogen monoxide syntetase
- OAT: ornitin-amino-transpherase
- ODC: ornitin-decarboxilase
- OKTPI: Korányi National Institute of Tuberculosis and Pulmonary Diseases
- PaCO₂: partial arterial carbon dioxide pressure
- PAF: platelet activating factor
- PaO₂: partial arterial oxygen pressure
- PE: phosphatidil-etanolamin
- PGI₂: prostaciklin
- PRMT: protein arginine metiltranspherase
- PS: phosphatidilserin
- Raw: airways resistance
- RV: residual volume
- SAA: serum amyloid-A
- SD: standard deviation
- SDMA: symmetrical dimethylarginine
- SGRQ: St. George's Respiratory Questionnaire
- SLPI: secretory leukoprotease inhibitor
- SPD: surfactant protein D
- TF: tissue factor
- TGV: thoracal gas volume (TGV=RV+ERV)
- TLC: total lung capacity
- TNF- α : tumor necrosis factor alfa
- TTP: thrombotic thrombocytopenic purpura
- vWF: von Willebrand factor
- WHO: World Health Organisation

1. Introduction

1.1. Reasons topic choice

Besides dr. Attila Kovács and dr. Viktor Vörös, dr. László Kovács inspired me to conduct research during my work in the Student Researchers' Society at university. I encountered patients' subjective complaints face-to-face for the first time during the treatment of chronic patients in his family physician's practice. My interest in chronic obstructive pulmonary disease was aroused by dr. Zoltán Balikó, who encouraged me to investigate COPD patients in the first years of my residency training. There has been many changes both in the management of COPD and in understanding the background of the disease in the 12 years that elapsed since then, but there are still many open issues remaining. In addition to the increase in the prevalence of COPD, the increase in the patient population and its ever more important role in the increasingly aging population, I have also witnessed individual human destinies and disease progressions in my professional career. Especially as the head of the Special Respiratory Care Unit of the Division of Pulmonology, I came in contact with the most severely ill COPD patients on a daily basis. Late dr. Tamás Magyarlaki encouraged me to investigate microparticles in this patient population and dr. Gitta Tökés-Füzesi aided me in its practical implementation, while dr. Tihamér Molnár opened up my mind to the importance of NO pathways in diseases associated with chronic hypoxemia and inflammation. Therefore, with my research results I aim to contribute to the earliest possible screening of these patients, early recognition of their acute exacerbations, commencement of adequate therapy, and a better understanding of the pathomechanisms of the disease. Nowadays, COPD is a leading cause of morbidity and mortality in developed countries. At a global level, COPD currently ranks between 4th and 6th places in the causes of death and is expected to be the third leading cause of death by 2020. This is caused by an increase in the environmental impacts damaging the respiratory system (mainly smoking), the aging population and improvement in the therapeutic outcomes in other major patient groups (e.g. cardiovascular patients). COPD constitutes a major social and economic burden due to its high prevalence and its chronic and progressive course. In the European Union, direct costs of respiratory diseases amount to 6 % of total healthcare costs, 56 % of which is allocated to the management of COPD. In the meantime, current pharmacotherapeutical options have a low efficacy on the progression of the disease, therefore prevention plays a major role in this patient population. Thus, in addition to markers used so far in clinical practice, it is important to determine new biomarkers, which aid in the assessment of factors involved in disease progression, in the early screening of patients at risk and in the follow-up of the individual course of the disease and the efficacy of the therapy.

1.2. Chronic obstructive pulmonary disease

1.2.1. Definition

Chronic obstructive pulmonary disease is a condition associated with slowly and gradually deteriorating dysfunction and increased bronchial flow resistance, which is not completely reversible, but the obstruction can slightly be reduced by bronchodilators and other therapies in the long run. Increased airflow resistance is due to an abnormal inflammatory response of the lungs induced by persistent inhalation of harmful gases or particles. The predominant symptom of chronic obstructive bronchitis is productive cough persisting for a minimum of three months a year in at least two consecutive years, which is not caused by a heart or other lung disease. Airway obstruction is constituted partly by peripheral bronchoconstriction (bronchial inflammation and restructuring of the bronchial walls) and partly by the emphysematous destruction of lung tissue (destruction of alveolar walls and reduced contractile force of the lungs). Chronic obstructive bronchitis and emphysema differ in their pathogenesis, but they typically co-occur and an individual patient can be characterized by the dominance of the bronchitic or the emphysematous component. Assessment of the quality of life of COPD patients is inevitable in clinical practice and objectivization of symptoms has recently gained a prominent role in grading the severity of the disease and selecting the adequate therapy. The COPD Assessment Test (CAT) and the Clinical COPD Questionnaire (CCQ), which are short, easy-to-use and can be completed by patients on their own, are both suitable for this purpose, as has been shown in one of our studies.

1.2.2. Epidemiology

Data relating to the prevalence of COPD are very heterogenous due to deviations in diagnostic criteria of the disease and methods of assessment of respiratory function. According to surveys published in industrialized countries, COPD currently affects 4-7 % of the adult population. Its prevalence is lower in females than in males but it increases at a faster rate in females. In Hungary, available data relating to COPD prevalence fall short of estimated data. The Hungarian registry mainly includes severe cases requiring hospitalization on a regular basis while milder cases, which are in majority and are more promising with regard to preventive and bronchodilator therapy are undetected and therefore untreated. If the Burden of Obstructive Lung Disease (BOLD-2007) trial results indicating a COPD prevalence of 9-10 % in adults are projected to the Hungarian population aged over 40, the number of Hungarian COPD patients staged GOLD II-III/IV can be approximately 5-6000, which is three times more than the patient population of 168431 registered in the pulmonary treatment centers in 2012. In Hungarian pulmonary care registry, the number of moderately ill COPD patients is the highest, while the percentage of diagnosed, mildly ill patients can amount to 23 %. It should be highlighted, though, that the number of COPD

patients is increasing year after year. In Baranya county, our colleagues treat the third largest population of registered COPD patients in Hungary. In Hungary, 34% of the adult population smoke on a regular basis. It should be highlighted that the percentage of smokers is increasing rapidly in the teenage and young female population; currently 40% of them smoke. In the developing countries, ca. 3 billion people burn coal or use biomass (wood, coal, manure) for cooking or heating. According to a Chinese survey, a group of never smoker rural females exposed to smoke from biomass had a three-times higher prevalence of COPD (7.2%) than a group of urban females not exposed to smoke from biomass. Similar observations have been carried out in the PLATINO trial in South America, which found a prevalence of the disease of 18.4-32.1% in the population aged over 60. However, COPD is not only a severe inflammatory disorder of the airways, but a complex clinical syndrome accompanied by systemic inflammation associated with considerable extrapulmonary components and some researchers consider the inflammation to be the underlying feature of the disease. Furthermore, the manifestation of the disease is strikingly multifaceted: patients classified into the same severity stage considerably differ in the course of disease, in the therapeutic response, in the severity of the experienced symptoms and in the speed of loss of respiratory function.

1.2.3. Acute exacerbation

According to the GOLD definition, exacerbation (AECOPD) is a prolonged event interfering with the natural course of disease. A change occurs in the underlying symptoms of the disease which exceeds the extent of normal daily fluctuations, develops acutely and requires modification of therapy. Exacerbations are severe, acute episodes associated with respiratory and systemic inflammation, acute intensification of symptoms and disruption of airflow and require supplementary therapy. Bacteria colonizing the upper airways (*H.Influenzae*, *S.Pneumoniae*, *M.Catarrhalis*) easily enter the lower airways due to damage to the airway epithelial barrier caused by cigarette smoke and colonize them. If the cell count of these bacterium colonies increases during progression of the disease, acute respiratory infection develops and the patient is exacerbated. This inflammation increases salivary secretion and damage to the respiratory epithelium and mucociliary clearance. Previous studies have found that acute exacerbations cause increase in respiratory and systemic inflammation, elevation in serum concentrations of C-reactive protein (CRP), fibrinogen and IL-6 and in the concentration of IL-6, IL-8, leukotriene B4 (LTB-4), myeloperoxidase (MPO), matrix metalloproteinase (MMP) in the sputum. Neutrophil and eosinophil counts also increase. In severe purulent exacerbation the bacterial count in sputum is elevated.

2. Objectives

- finding out whether (i) the amount of platelet-derived, endothelial cell-derived, monocyte-derived and erythrocyte-derived microparticles (MP) increases, (ii) these MPs can be used as biomarkers to follow-up the severity of disease, (iii) these MPs are suitable for differentiating exacerbation from stable disease
- determination of differences in the microparticle levels in COPD patients with cardiovascular co-morbidities (CVD) and those without cardiovascular co-morbidities
- determination of differences in the microparticle levels in COPD patients with cardiovascular co-morbidities (CVD) and those without cardiovascular co-morbidities, with regard to the presence or absence of acute exacerbations
- investigation of the effect of smoking on microparticle levels in the above-mentioned COPD groups
- prospective investigation of markers of L-arginine pathway (L-arginine, ADMA, SDMA) in the sera of stable and exacerbated COPD patients and in those of healthy subjects
- exploration of the link between the classic infection marker CRP and markers of L-arginine pathway
- retrospective follow-up (5 years): auditing the number of events with exacerbations and then investigating the prediction value of the studied markers
- investigation of the link between treatment with beta adrenergic receptor blockers (BB) and the prevalence of COPD exacerbation
- finally: comparison of molecules of L-arginine pathway and CRP levels in BB-treated COPD patients and in those not receiving BB treatment

3. Microparticles and COPD

3.1. Microparticles

3.1.1 Microparticles in general

Microparticles (MPs) found in the circulation are vesicles which are generated by budding off cell membranes through cell activation or apoptosis. They are present in the blood physiologically, however, their level in the blood may change through pathophysiological processes. Wolf described them as platelet-derived procoagulant particles first in 1967. The majority (70-90%) of MPs derives from platelets, however, they can also derive from leukocytes (neutrophil granulocytes, lymphocytes, monocytes, macrophages), erythrocytes, endothelial cells or smooth muscle cells. On average, the size of MPs ranges from 0.1 to 1.0 μm . Particles below 0.1 μm are called exosomes, which enter the extracellular space intracellularly from multivesicular bodies of endosomal origin. Meanwhile, fragments deriving from apoptotic cells (apoptotic bodies) exceed the average size of MPs ($<4.0 \mu\text{m}$). The membranes of MPs are abundant in lipids and proteins. Their phospholipid composition is different from that of the cell they derive from. The outer layer of their dual membrane mainly contains phosphatidylserine (PS) and phosphatidylethanolamine (PE), which are negatively charged amino phospholipids. They carry superficial antigens/proteins specific to their cell origin which enable their identification.

3.1.2. The role of microparticles

Regarding their function, they are involved in intercellular communication, the protection of cells and transmission of genetic information. They are concerned in inflammatory processes, activation of the coagulation system and certain vascular functions. MPs play a part in intercellular communication by transporting receptors, cytokins and messenger molecules. On the one hand, they can play the role of circulating signal molecules which affect features of the target cell by carrying bioactive molecules which can activate their receptors. On the other hand, they are able to activate cells and affect cell functions by transporting proteins, lipids and RNA fragments and by fusing with cells. As MPs can be found not only in the vicinity of the cell they derive from but also in the whole circulation, they can play a part in remote signal transmission communication. Various receptors (GP IIb/IIIa, GP Ib, P-selectin/CD62P) which can activate endothelial cells, leukocytes and monocytes can be found on the surfaces of platelet-derived MPs. Arachidonic acid appearing on MPs can also trigger cell activation. MPs generated by oxidative stress to the cells mainly contain oxidized phospholipids, which can be eliminated by the cells with increased production of MPs. They can be involved in the transmission of genetic information through their ability to transfer mRNA and microRNA.

Platelet-derived microparticles can induce an inflammatory process by transferring arachidonic acid to endothelial cells, which triggers increased CD54 expression, promoting adhesion of leukocytes to the vessel walls. Oxidative stress developing in inflammatory processes and apoptosis cause oxidized phospholipids to appear on the surfaces of MPs, thereby promoting adhesion of monocytes to endothelial cells and activating neutrophil cells. These oxidized phospholipids induce activation by PAF (platelet activating factor) on endothelial cells and leukocytes. PS found on the surfaces of platelet-derived MPs play an important part in the development of coagulation, as coagulation factors bind to PS (IXa, VIII, Va and IIa) in the presence of Ca^{2+} in this process, leading to the formation of tenase and prothrombinase complexes and eventually resulting in thrombin formation. P-selectin and TF (tissue factor) expressed on the surfaces of platelet-derived MPs also have a prothrombic effect. Endothelial cell-derived MPs express large von Willebrand factor multimers, thus being able to induce platelet aggregation and increase the stability of the aggregate. Regarding their vascular function, they transport arachidonic acid to the endothelial cells, where cyclooxygenase-2 (COX-2) induces prostaciline (PGI₂) formation, which has a vasodilator effect and can reduce the reactivity of platelets, thus protecting the vessels and reducing the risk of development of coagulation.

3.2. Patients and methods

3.2.1. Objectives of the investigation

In our study, the amount of microparticles measurable in COPD patients' peripheral blood was investigated according to the markers determining their origin and results of exacerbated patients were compared with those without exacerbations. In our objectives, we tried to find out whether the amount of platelet-derived, endothelial cell-derived, monocyte-derived and erythrocyte-derived microparticles increases as a result of inflammatory processes in COPD and whether they can be used as biomarkers to explore and follow up the disease and its complications.

3.2.2. Patients

Patients suitable for the study were selected from the pulmonary outpatients' clinic, patients presenting to the County Pulmonary Treatment Center for follow-ups and in-patients previously treated in the division of pulmonology. In the case of exacerbated patients, sampling was taken in the morning following admission. The study protocol was approved by the Regional Research Ethics Committee of the University of Pécs, Medical School

(27.03.2009., 3429). After receiving information, the patients assented to the study by signing the consent form.

In the study, several biomarkers were investigated in these three patient groups and their results were compared with those of the healthy control group:

- **COPD:** stable COPD patients without CVD
- **COPD+CVD:** stable COPD patients with coexisting CVD
- **AECOPD:** COPD patients with acute exacerbations without CVD and **AECOPD+CVD:** COPD patients with acute exacerbations with coexisting CVD

Past histories of COPD patients with cardiovascular co-morbidities (CVD) include myocardial infarction, positive coronarography, vascular surgery, cerebrovascular event and peripheral vasoconstriction. 20 patients were enrolled into each group and a total of 60 patients and 20 voluntary controls were examined. The patients were aged between 32-84 and the male-female ratio was 2:1. The average age was 63.2. The average age in females was 71.1 while the age of males averaged 58.2. The number of smokers was 26 altogether. Volunteers presenting to an ophthalmological operation who were of similar age to members of our patient groups and had no co-morbidities were included in the control group.

3.2.3. Investigated parameters

Post-bronchodilator FEV1 value was determined with spirometry in patients to confirm the diagnosis of COPD and assess the stage of disease. In addition, a blood test was done to help determine the following parameters and biomarkers:

- CRP
- leukocyte count
- total MP count, endothelial cell-derived, platelet-derived, erythrocyte-derived and leukocyte-derived MP count (A+MP, A+CD41, A+CD61, A+CD42a, A+PAC1, A+CD31, A+CD62E, A+CD45, A+CD13, A+CD14, A+GlyA)

3.2.4. Sampling

Anticoagulated whole blood was used to isolate circulating microparticles. Sampling for the flow cytometry was collected from the antecubital vein with a 21G needle using vacutainer tubes containing Na₃-citrate as anticoagulant. The samples were processed within an hour of sampling. K₃-EDTA tubes were used to determine blood count and hs-CRP was determined using native (not containing anticoagulants) tubes. The measurements were done in the Department of Laboratory Medicine, UPMS.

3.3. Results

3.3.1. COPD vs. COPD+CVD patient groups

In the group of patients without co-morbidities, exacerbated patients were found to have a significantly higher level of hsCRP (mg/l) than those without exacerbations. Comparing the results of the group of stable COPD patients with coexisting CVD (COPD+CVD) with those of the group of COPD-only patients with exacerbations (AECOPD), significantly higher values were measured in the latter group. More elevated levels of hsCRP values shown in the group of exacerbated COPD patients with coexisting CVD were not significantly higher compared to the subgroups of stable patients and the group of COPD-only patients with exacerbations. Significantly more elevated white cell counts (G/l) were detected in the group of exacerbated COPD patients without co-morbidities (AECOPD) and in the group of exacerbated COPD patients associated with co-morbidities (AECOPD+CVD) compared to the stable COPD-only group and in the group of COPD+CVD patients. The stable and the exacerbated subgroups had significantly elevated absolute microparticle counts compared with the control group. Comparing the COPD subgroups to each other, no significant differences were measured between the individual groups. Both platelet-derived CD61 and CD41 markers were significantly elevated in both the stable and the exacerbated subgroups, compared with the control group ($p < 0.001$). There were no significant differences between the individual COPD subgroups. With regards to platelet-derived CD42a marker, there was a significant elevation only in the group of exacerbated COPD patients without coexisting CVD (AECOPD) compared with the control group, but not in the other subgroups. No significant differences were detected between the individual COPD subgroups. With regards to the PAC1 platelet activation marker, no significant differences could be evaluated due to the high standard deviation. No significant deviations in the endothelial cell-derived CD31 marker were measured in any of the subgroups, compared with the control group and each other. A significant elevation in the endothelial cell activation marker CD62E was observed in both the subgroups of stable COPD patients and in the subgroups of exacerbated patients compared with the control group. No significant differences were detected between the four COPD subgroups. Both the subgroups of stable COPD patients and the subgroups of exacerbated patients had significantly elevated levels of leukocyte-derived CD45 and CD13 markers compared with the control group, but no significant differences were detected between the four COPD subgroups. In the case of platelet-derived CD14 marker, there was significant elevation only in the group of exacerbated COPD patients associated with coexisting CVD (AECOPD+CVD) compared with the control groups, while no such deviation was observed

in the other subgroups, compared with the control group. No significant differences were detected between the individual COPD subgroups. In the case of erythrocyte-derived Glycophorin A (CD235) marker, a significant elevation can be observed in the group of stable COPD patients with coexisting CVD (COPD+CVD), in the group of exacerbated COPD patients without coexisting CVD (AECOPD) and in the group of exacerbated patients with coexisting CVD (AECOPD+CVD) compared with the control group. No significant differences were found between the individual COPD subgroups.

3.3.2. Comparison of platelet-derived, endothelial cell-derived and monocyte-derived markers in patients with stable COPD (n=40) vs exacerbated patients (n=20)

On investigation of the stable (COPD) group and the exacerbated patient group (AECOPD) not taking CVD into account, significantly elevated levels of CD42a, CD62E and CD14 markers were found in the latter group. Both the stable group and the exacerbated patient group had significantly elevated values compared with the control group. When comparisons were made with regards to smoking, no significant differences were found between the COPD-only subgroups or between the CVD subgroups. No significant deviations were found, either, when aggregating the COPD-only groups and the COPD-CVD groups.

3.3.3. Summary of the results

Stable COPD vs control: When investigating stable COPD patients, significant differences were found in the amount of almost all microparticles compared with the control group.

AECOPD vs control: In exacerbated patients, significantly more elevated levels of absolute microparticles, platelet-derived CD61 and CD42a markers (in the case of the latter: only in COPD-only exacerbations), endothelium-derived CD62E marker, leukocyte-derived CD45 and CD13 markers, monocyte-derived CD14 marker (only in exacerbations associated with coexisting CVD) and in erythrocyte-derived glycophorin A marker. No significant deviations were detected in platelet-derived PAC1 and in endothelium-derived CD31 markers compared with the control group.

Stable COPD vs AECOPD: Between the stable group and the exacerbated patient group, significant differences were shown in CD42a (platelet-marker), CD62E (endothelial cell marker) and CD14 (monocyte marker) values. In the exacerbated patient group, significantly more elevated levels of inflammatory markers (CRP, leukocyte) were detected compared with patients with stable COPD.

COPD+CVD vs COPD-only: No significant differences were found in the microparticle levels of COPD-CVD patients and those of COPD patients without CVD in the stable COPD patient group and in the exacerbated patient group. When comparisons were made

with regards to smoking, no significant differences were found between the group of patients without co-morbidities and the groups with coexisting CVD.

4. L-arginine pathway and COPD

4.1. L-arginine pathway in general

During the metabolism of L-arginine, nitrogen oxide, L-citrulline, urea and L-ornithine are formed in the NO-synthase (NOS) and arginase pathways. L-ornithine is broken down to polyamines by ornithine decarboxylase (ODC), which blocks NOS activity, or by ornithine aminotransferase (OAT), which forms proline, a precursor in collagen biosynthesis. L-arginine is built in proteins, during which protein arginine methyltransferase (PRMT) produces monomethylized or dimethylized forms, releasing monomethyl arginine (MMA) and asymmetric dimethylarginine (ADMA), which is an endogenous competitive inhibitor of NOS, by proteolysis. This process also releases symmetric dimethylarginine (SDMA), which is a competitor of L-arginine preceding cationic amino acid transporter 2. There is a dual connection between hypoxia and L-arginine - nitrogen monoxide pathway. Hypoxia not only induces overproduction of NO but also promotes arginine-methylation of proteins. During the production of NO, nitrogen monoxide synthase aids the formation of L-citrullin from L-arginine. Renal function plays a major part in decreasing the level of SDMA, whereas ADMA is metabolized in the liver. Three NOS isoforms have been identified, endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). While eNOS and nNOS are mostly calcium/calmodulin dependent, iNOS can be produced as a result of hypoxia or an inflammatory process. NOS activity is reduced by certain endogenous inhibitor molecules, e.g. asymmetric dimethylarginine (ADMA).

4.2. Objectives

The aim of the study was a prospective investigation of L-arginine pathway markers (L-arginine, ADMA, SDMA) in the sera of stable and exacerbated COPD patients and in healthy subjects. We also aimed to explore the link between classic CRP infection marker and L-arginine pathway markers. In the second main section of the study, which is a retrospective investigation, the number of exacerbation events were audited during a 5-year follow-up to gain information on the predictive force of the investigated markers. In a recently published article it was implied that pre-treatment with beta-adrenergic blockers (BB) may be linked to a significant decrease in COPD exacerbations. Therefore, it seemed reasonable to compare

asymmetric and symmetric dimethylarginine (ADMA, SDMA) and hsCRP levels in the sera of BB-treated COPD and in those not treated with BB.

4.3. Patients and methods

The study protocol was approved by the Regional Local Ethical Committee of the Medical School, University of Pécs (3950.316-17030/KK4/2010). The patients enrolled into the study signed a consent form. A blood sample was collected from 30 healthy subjects from the control group and their ages were indicated.

4.3.1. Patients

A total of 44 COPD inpatients and outpatients of the Division of Pulmonology, 1st Department of Medicine, University of Pécs, were selected into our prospective study in 2010. All of the patients met the diagnostic criteria for COPD, as their FEV1/FVC values were below 70%. Their severity staging was based on the GOLD staging system, version of 2010: I = mild: $FEV1 \geq$ reference 80%, II= moderate: reference 50% \leq FEV1 < reference 80%, III=severe: reference 30% \leq FEV1 < reference 50%, IV=very severe: FEV1 < reference 30% or FEV1 < reference 50% associated with chronic respiratory failure. COPD exacerbations were based on GOLD guidelines: exacerbation of the extent of underlying dyspnea, exacerbation of cough/ productive cough deviating from the normal daily fluctuations, occurrence of acute attacks requiring modification of previous medication regimen, and we added the criteria of the subject requiring hospitalization (in the division of pulmonology or emergency care). In the second stage of the study, in 2016, we collected retrospectively the number of exacerbations of all patients enrolled into the study from our on-line documentation (UP-CC, eMEDsol database) during the year prior to and the 5 years following sampling. Exclusion criteria included chronic renal failure (eGFR < 50ml/min/1.73m² and/or serum creatinine >120 μ mol/l), a diagnosed malignancy and rejection of participation in the study. Questioning included a history of coronary artery disease, myocardial infarction, diabetes mellitus, hypertension, dyslipidemia and stroke, current smoking habits and home medication regimen, with special regard to taking beta adrenergic blockers. In accordance with our clinical practice, a basic capillary blood gas test was done in each patient.

4.3.2. Determination of L-arginine derivatives and hsCRP

A fasting venous blood sample was collected from stable COPD patients presenting to our outpatients' clinic and from patients hospitalized due to COPD exacerbations in the morning

the day following their admission. The serum sample was frozen to minus 70 C° until the analysis was carried out. Serum hsCRP was measured using automated fluorescence immunoassay (BRAHMS Kryptor, Hennigsdorf, Germany). To measure L-arginine, ADMA and SDMA, the amino acid content of the blood sample was obtained with a solid-phase extraction (SPE) method and was measured using high-performance liquid chromatography (HPLC).

4.4. Results

4.4.1. Comparison of the COPD group and the healthy subject group

The L-arginine level was significantly more elevated in the 44 patients enrolled into the study compared with the healthy control group ($p < 0.05$). ADMA and SDMA levels were also significantly more elevated compared with the healthy control group ($p < 0.001$). The serum hsCRP level was also significantly higher in COPD patients ($p < 0.05$).

4.4.2. AECOPD vs stable COPD

Significantly more elevated levels of ADMA and SDMA were detected in the AECOPD group compared with stable COPD patients, however, no significant differences were found in the L-arginine and hsCRP levels. Based on a ROC analysis, ADMA (cut-off $\geq 0.69 \mu\text{mol/l}$, AUC: 0.81, $p = 0.001$) discriminated the AECOPD group from the stable COPD group with 75% sensitivity and 73% specificity whereas SDMA (cut-off $\geq 0.57 \mu\text{mol/l}$, AUC: 0.91, $p < 0.001$) discriminated AECOPD patients from stable COPD patients with 92% sensitivity and 80% specificity. Based on a multiple regression analysis, SDMA serum concentration $\geq 0.57 \mu\text{mol/l}$ can independently (OR: 1.632, $p = 0.001$) discriminate AECOPD patients from stable COPD patients. The capillary pO₂ level was significantly lower in the AECOPD group compared with stable COPD group ($p = 0.01$). Capillary pO₂ levels indicating the severity of hypoxia were shown to be significantly negatively correlated with serum ADMA levels ($p < 0.05$).

4.4.3. COPD patients with respiratory failure

Oxygen saturation below 90% was detected in 16 patients. Significant differences were found in neither L-arginine serum levels nor the serum levels of its derivatives (ADMA, SDMA) between COPD patients with respiratory failure and those without respiratory failure.

4.4.4. The frequency of exacerbations - retrospective data collection

The number of exacerbations of hospitalized stable COPD patient enrolled into the study was also investigated in the year prior to and the first and 5th year following sampling. A retrospective examination of stable patients the year prior to sampling detected a significantly lower L-arginine level in patients with one or more exacerbations (8 subjects) compared with those without exacerbations (median: 91.90 $\mu\text{mol/l}$, IQR: 83.90-102.50 vs. 111.70 $\mu\text{mol/l}$, IQR:100.20-122.20, $p=0.02$). Investigating the year following sampling, we found that COPD patients who were stable at sampling but later developed exacerbations ($n=2$) also had a lower L-arginine level compared with the other patients, however, this deviation was not proved to be significant ($p=0.05$). Furthermore, this deviation was not detected in the data obtained during the 5-year follow-up. Investigating 5-year survival, stable patients who survived for 5 years ($n=2$) were shown to have a significantly lower hsCRP level than those who did not survive for 5 years (median: 6.00 mg/l, IQR: 3.20-61.00 vs. 15.97 mg/l, IQR: 10.09-29.30, $p=0.028$).

4.4.5. The role of therapy with beta adrenergic blockers in changes in the investigated parameters and the frequency of exacerbations

In the retrospective stage of the study, stable and exacerbated patients were grouped according to use of beta adrenergic blockers (BB). In the subgroup of BB pre-treated patients, AECOPD patients had a significantly more elevated serum hsCRP compared with stable COPD patients (median: 30.04mg/l, IQR: 15.04-39.92 vs. 5.60mg/l, IQR:3.69-10.32 $p=0.021$). Investigating the link between hsCRP and BB treatment, we found that in COPD exacerbation BB-treated patients had a significantly lower serum hsCRP compared with patients not on BB treatment (median: 3.3, IQR:2.32-7.4 vs. 30.04, IQR:15.04-39.92, $p=0.01$). In the subgroup of BB pre-treated exacerbated COPD patients, ADMA was more elevated (median: 0.93, IQR:0.85-0.97 vs. 0.69, IQR:0.64-0.78, $p=0.06$) than in exacerbations without BB, however, this difference was not significant, probably due to the small sample size. Both ADMA ($p=0.003$) and SDMA ($p< 0.001$) were significantly more elevated in exacerbated patients than in stable patients when BB treatment was not taken into account. This difference remained significant when patients were pre-treated with BB. During the follow-up of all investigated patients, BB pre-treatment was not shown to significantly correlate with the number of exacerbations in the follow-up period, and it was not found to have an effect on 5-year mortality, either. Interestingly, hsCRP taken in stable condition correlated positively with the number of exacerbation ($p=0.003$) only in the subgroup not receiving BB pre-treatment

5. Summary of novel results

1. We have proved that stable COPD patients had a significantly more elevated absolute microparticle count and the amount of microparticles indicated with platelet-derived CD61 and CD41, endothelium-derived CD62E and leukocyte-derived CD13 markers compared with the healthy control group, and this deviation was also found in cases associated with CVD and in cases not associated with CVD.

2. We have proved that in the systemic circulation of AECOPD patients there was a significantly more elevated absolute microparticle count and MP levels indicated with platelet-derived CD61 and CD41, endothelium-derived CD62E and leukocyte-derived CD 45 and CD13, platelet-derived CD 14 and erythrocyte-derived glycophorin A markers, and this deviation was also seen in cases associated with CVD and in cases not associated with CVD.

3. Significant differences in CD42a (platelet marker), CD62E (endothelial cell marker) and CD14 (monocyte marker) have been detected between the stable and the exacerbated patient groups, more elevated values having been found in the latter group.

4. We have proved that the serum levels of methylated arginine derivatives (ADMA, SDMA) and precursor L-arginine molecule are more elevated in COPD patients than in healthy subjects.

5. Furthermore, we have shown that the serum levels of these molecules are even more elevated in COPD exacerbations.

6. We have demonstrated that a low L-arginine level is linked to a higher frequency of COPD exacerbations.

7. Interestingly, a positive correlation between hsCRP and the number of exacerbations ($p=0.003$) was detected only when it was obtained in stable COPD in the subgroup of patients not receiving beta adrenergic blockers. According to our own data, hsCRP measured in systemic circulation in patients receiving beta adrenergic blockers is less informative in monitoring exacerbation.

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7.List of publications

7.1.The thesis is based on the following papers

- 1 . **Ruzsics I**, Nagy L, Keki S, Sarosi V, Illes B, Illes Z, Horvath I, Bogar L, Molnar T. L-Arginine Pathway in COPD Patients with Acute Exacerbation: A New Potential Biomarker. COPD 2016;13:139-45.**IF:2,16**
2. **Ruzsics I**, Biri B, Nagy L, Kéki S, Sárosi V, Horváth I, Bogár L, Molnár T. Metilált arginin származékok szerepe a stabil és az exacerbált COPD betegeknél: követéses vizsgálat. Medicina Thoracalis, 2017;70: 3-12.

Cumulative IF of original papers in peer-reviewed journals: 2,16

7.2. The thesis is based on the following presentations (citable abstracts)

1. A CRP jelentősége COPD acut exacerbatiojában (poszter)Magyar Tüdőgyógyász Társaság 54. Nagygyűlése, 2006.06.08-10, Szeged, Absztrakt: Medicina Thoracalis – 2006, Suppl.69.o.
2. Biomarkerek (mikropartikulumok, gyulladásos markerek) vizsgálata COPDs betegeink körében. Pulmonológus Kutatók Fóruma, 2012.06.13, Budapest.
3. COPD and the microparticles. Croatian–Slovenian–Hungarian, Pulmonologist Meeting, Siófok, 2011 Ápr. 7-10,
4. Gyulladásos és thrombocytá aktivációs markerek vizsgálata COPDs betegeink körében. Magyar Tüdőgyógyász Társaság 57. Nagygyűlése és 100 éves Centenárium Emlékülése, 2012. június 13-16, Budapest.
5. A mikropartikulumokról a COPD és a társult kardiovaszkuláris betegségek kapcsán. A Magyar Hypertonia Társaság 11. Nemzetközi Továbbképző Előadás sorozata és 20. Kongresszusa, 2012.dec.9-12, Budapest,
6. Increased circulating microparticles in COPD patients. 3rd Croatian – Slovenian – Hungarian Pulmonologist Meeting, 18. Apr. 2013, Brijuni, Croatia
7. Microparticles in stable and exacerbated COPD patients (poster, Silver Sponsorship) ERS Congress, 07-11, Sept. 2013, Barcelona, Spain
Eur Respir J 2013 42:Suppl 57, P777, **IF:7,125**
8. Methylated arginine derivatives can differ stable and unstable patients with COPD.ERS Congress, 07-11, Sept. 2013, Barcelona, Spain
Eur Respir J 2013.42: Suppl57,394, **IF:7,125**
9. Microparticulum vizsgálataink COPD-s betegek körében. Pulmonológia Akadémia, Hajdúszoboszló, 2013.09.21.

10. Biomarker kutatás Pécsen COPD exacerbációban. Fókuszban a beteg - Országos Tudományos Rendezvény, Pécs, 2014.05.17
11. Methylált arginin származékok szerepe a stabil és exacerbációban lévő COPDs betegek differenciálásában. Magyar Tüdőgyógyász Társaság 58. Nagygyűlése, 2014.június, Medicina Thoracalis, LXVII. évfolyam, 2014 június, 190.o.
12. L-arginine pathway metabolites in COPD patients with and without acute exacerbation: a follow-up study, Hungarian Medical Association of America, Summer Conference Balatonfüred, 2016 08. 27.
13. L-arginine pathway metabolites can predict exacerbation independently from beta blocker treatment in patients with COPD: A follow-up study. ERS Congress, 03-07, Sept, 2016 London, UK
Eur Respir J 2016 48 Issue Suppl 60, PA996, **IF:10,569**
14. Az L-arginin útvonal összefüggése a COPD exacerbációjának gyakoriságával: egy 5 éves utánkövetés eredményei. Magyar Tüdőgyógyász Társaság 59. Nagygyűlése, Debrecen, 2016. június 8 - 11.
15. Gyulladás jelentősége COPD-ben, Előadás, 22.Tavaszi AMEGA Fórum, 2017.03.24, Pécs
16. Változások a GOLD 2017/18-ban, Előadás, 1. Rehabilitációs Kazuisztikai Fórum, 2018.02.09-10, Sikonda

Cumulative IF of citable publications: 24,819

Number of citable abstracts: 3

7.3. Other publications

1. **Ruzsics I**, Hegedűs G, Agócs Á, Komoly S, Sinkovicz A, Sárosi V. A kép és valóság. Medicina Thoracalis. 2007;60:314-8.
2. Sárosi V, **Ruzsics I**, Enyezdí J, Grexa E, Balikó Z. Az Avastin kezelés biztonságossága egy tüdő-adenocarcinomás beteg kezelése kapcsán. Tüdőgyógyászat 2009; 3:24-25.
3. Sárosi V, Balikó Z, Smuk G, Szabó M, **Ruzsics I**, László T, Mezősi E. The frequency of EGFR mutation in lung adenocarcinoma and the efficacy of tyrosine kinase inhibitor therapy in a Hungarian cohort of patients. Pathol Oncol Res. 2016;22:755-761.**IF:1,94**
4. Matancic M, Faludi B, Sárosi V, **Ruzsics I**. Tartós otthoni NIV kezelés – Egy összeadott élet. Medicina Thoracalis. 2016;69:306-308.

5. Jakab Z, Szabó M, **Ruzsics I**, Balikó Z, Sárosi V. A CAT (COPD Assesment Test) és a CCQ (Clinical COPD Questionnaire) életminőség kérdőívek jelentősége a COPD-ben szenvedő betegek keresztmetszeti állapotfelmérésében, *Medicina Thoracalis*, 2015; 68:264-269
6. Vörös V, Osváth P, **Ruzsics I**, Nagy T, Kovács L, Varga J, Fekete S, Kovács A. Az öngyilkos viselkedés gyakorisága és jellegzetességei a családorvosi gyakorlatban. *Orvosi Hetilap*. 2006;147:263-268.
7. Vörös V, Osváth P, **Ruzsics I**, Varga J, Kovács L, Fekete S, Kovács A. Prevalence of mental disorders among psychiatric drug users in general practice. Poster, 18th ECNP Congress, abstract published in *European Neuropsychopharmacology (Suppl)*2005;15:S556-S556, **IF:3,510**
8. Müller V, **Ruzsics I**, Balikó Z. A HERMES programról. *Medicina Thoracalis*, 2007;60:47-52.
9. Vörös V, Osváth P, **Ruzsics I**, Varga J, Kovács L, Fekete S, Kovács A. Prevalence and treatment of mental disorders in general practice. Annual Scientific Meeting European Association for Consultation-Liaison Psychiatry and Psychosomatics (EACLPP). *Journal of Psychosomatic Research*, 2005;59:48-50. **IF:2,052**
10. Vörös V, Osváth P, **Ruzsics I**, Nagy T, Kovács L, Varga J, Fekete S, Kovács A. A pszichotróp gyógyszerhasználat, az affektív betegségek és a szuicid viselkedés gyakoriságának és jellegzetességeinek felmérése egy háziiorvosi körzetben. *Neuropsychopharmacologia Hungarica* 2005;7 (suppl.):52.
11. Vörös V, Osváth P, **Ruzsics I**, Nagy T, Kovács L, Varga J, Fekete S, Kovács A. Az öngyilkos viselkedés felmérése egy családorvosi körzetben. *Psychiatria Hungarica* 2005;20(suppl):105.

Cumulative IF of other publications: 7,502

Cumulative IF of all publications (excluding citable abstracts): 9,662

7.4. Other scientific presentations

1. Kép és Valóság (előadás, I. Díj). Fialat Pulmonológusok Kazuisztikai Fóruma, Budapest, 2006.12.08.
2. Image and reality (lecture). 7th Fenno-Ugric Conference on Pulmonology, Kuressaare, Estonia, 2007.05.10.
3. Sikeres bevacizumab kezelések tüdő adenocarcinómában (poszter, III. Díj). Magyar Tüdőgyógyász Társaság 55. Nagygyűlése, Balatonfüred, 2008.06.04-07.

4. NSCLC ellenes kezelések figyelemreméltó thromboticus mellékhatásai (poszter).Magyar Tüdőgyógyász Társaság 55. Nagygyűlése, Balatonfüred, 2008.06.04-07.
5. Alsó légúti mintákból nyert baktériumok fluorokinolon rezisztenciája kórházi beteganyagunkban (előadás).Magyar Tüdőgyógyász Társaság 55. Nagygyűlése, Balatonfüred, 2008.06.04-07.
6. Elégséges terápia-e a célzott antibiotikus kezelés kábítószer-fogyasztó egyén staphylococcus okozta pneumoniájában? (előadás, III.Díj)Fiatall Pulmonológusok Kazuisztikai Fóruma, Hajdúszoboszló, 2009 március 19-21.
7. Cured Small Cell Lung Cancer (előadás). II. Román-Magyar Tüdőgyógyász Találkozó, Szeged, 2009 11.06-08.
8. A pro Gastrin Releasing Peptide (ProGRP) tumormarkerrel szerzett tapasztalataink kissejtes tüdőcarcinómában (poszter).Magyar Onkológusok Társaságának 28. Kongresszusa, Budapest, 2009 november 12-14. Magyar Onkológia Suppl.2009;53:14.
9. Hodgkin-lymphomából gyógyult fiatal nőbeteg mesotheliomájának története (előadás).A Magyar Tüdőgyógyász Társaság Onkopulmonológiai Szekciójának Konferenciája, Tapolca, 2009. december 3 - 5.
10. Sürgősségi Sarcoidosis (előadás). Magyar Tüdőgyógyász Társaság 56. Nagygyűlése, Sopron, 2010. június 2-5. Medicina Thoracalis, 2010;63:216.
11. Solid tumor patients with symptomless pulmonary embolism recognized by restaging chest CT (poster).12th Central-European Lung Cancer Conference (CELCC), Budapest. 2-4. Dec.2010.
12. A tüdőrák, mint stigma (legjobb poszter díj)A Magyar Tüdőgyógyász Társaság Onkopulmonológiai és Légzésrehabilitációs szekció konferenciája, Sopron, 2011 12.01-03.
13. TBC a pulmonológus szemével (előadás). IX. Pécsi Reumatológus Rezidens és Szakorvosjelölt Fórum, Pécs, 2011 03.25.
14. Látens Tuberculosis – Az anti-TNF terápiaik vonatkozásában (előadás). Regionális Szakfőorvosi értekezlet, Pécs, 2011 05.12.
15. Pulmonológiai betegség extrapulmonalis tünetekkel (előadás).56. Dunántúli Belgyógyász Vándorgyűlés, Siófok, 2011 05.28.
16. Változások az egyedi tételes gyógyszerek elszámolásával kapcsolatban - Pulmonológus szemmel. Lilly szimpózium, Pécs, 2012 05.16.

17. Felnőtt betegek NIV kezelésének a körülményei (előadás). NIV Workshop orvosoknak, Budapest, 2012 10.12-13.
18. A 2012-es ERS üzenetei: NIV (előadás). A Magyar Tüdőgyógyász Társaság Továbbképző Tanfolyama, Budapest, 2013 01.25-26.
19. Leptomeningeális carcinosis vagy posterior reverzibilis encephalopathia szindróma? Avastin kezelés mellett (előadás). MTT Onkopulmonológiai Szekciójának konferenciája, Budapest, 2013 11.28-30.
20. Ritka, de életveszélyes mellékhatás Avastin mellett (poszter). Magyar Onkológusok Társasága 30. Kongresszusa, Pécs, 2013 11.14-16.
21. Pertussis, mint a collapsus oka (előadás). Magyar Tüdőgyógyász Társaság Továbbképző Tanfolyama, Budapest, 2016.01.29-30
22. Non-invazív ventiláció helye, szerepe. A Magyar Tüdőgyógyász Társaság COPD Világnapi rendezvénye és Újdonságok a Tüdőgyógyászatban rendezvény, Budapest, 2016.11.18-19.
23. Pertussis whooping cough as a cause for collapse. 4th Meeting of three Respiratory Societies: Slovenia, Croatia, Hungary, 22-23 May 2015, Bled, Slovenia
24. Fenntartó terápia elvei tüdőrákban. 'Onkológiai, Onko-pulmonológiai problémák a mindennapi gyakorlatban' továbbképző Lilly szimpózium, Pécs, 2014 10.13.