Original article:
The role of insertion-deletion polymorphism of the ACE gene in development of arterial hypertension in patients with chronic obstructive pulmonary disease
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Abstract:
Objective. This study aims to establish the role of insertion-deletion polymorphism of the angiotensin-converting enzyme gene in development of arterial hypertension in patients with chronic obstructive pulmonary disease. Materials and Methods: The study group consisted of 96 patients: Group 1 (25 individuals with COPD), Group 2 (23 individuals with AH), Group 3 (28 individuals with COPD and AH). The control group consisted of the 20 healthy subjects. I/D genotypes of ACE were determined by polymerase chain reaction (PCR) amplification. Plasma ACE activity was determined photometrically by a commercially available kit. Results and Discussion: The distribution of polymorphic variants of the ACE gene among COPD-only patients genotype spreading was close to the data obtained in controls. In hypertensive patients, there were fewer ID heterozygotes and more II homozygotes compared to controls. In the COPD+AH category of patients, II genotype was predominant in 7.1% subjects, DD genotype was predominant in 10.0% subjects and the proportion of ID heterozygotes was 17.1% lower compared to controls. The II genotype had a positive relationship with patient age and a negative relationship with body weights and respiratory rates of COPD+AH patients. The ID genotype was associated with increased respiratory rates; however, its correlation with the duration of the disease was negative. Conclusion: The data obtained in the study allow suggesting that polymorphism of the ACE gene doesn’t relate to development of AH in patients with COPD. The highest activity of ACE was found in patients with combination of COPD and AH; maximum findings of ACE activity were seen in patients with DD genotype.
Keywords: Chronic Obstructive Pulmonary Disease; Arterial Hypertension; Angiotensin-Converting Enzyme Gene; Insertion-Deletion Polymorphism.

Introduction
Chronic obstructive pulmonary disease (COPD) is defined by the American Thoracic Society as a progressive and partially reversible disease of respiratory tract featured by the limitation of airflow that takes place as a result of chronic bronchitis or emphysema. COPD is referred as a serious medical and socio-economic issue and the WHO predicts that by 2030 it will become the third most common cause of population mortality in the world. There are several established risk factors of COPD, of which tobacco smoking is the strongest one. Indoor air pollution, occupational exposures to coal dust, silica and asbestos, low birth weight, and recurrent...
infections are also considered as risk factors of COPD. There is a great disparity of COPD prevalence rates across countries worldwide. Global COPD prevalence is 9.2%, with 13.4% in Latin America compared to 6% in the US and 8.6% in Japan. The prevalence of COPD in Europe has been estimated to range between 4% and 10%, but in fact, of the 50 sovereign European countries, only 19 (38%) have reliable data on COPD prevalence available. Ukraine is characterized with extremely high COPD mortality rates compared with other European countries: while number of deaths per 100,000 of population is below 20 in Greece, Sweden, Iceland and Norway, it is more than 80 in Ukraine. Despite high mortality rates, very limited epidemiological COPD data are available in Ukraine. The CORE study conducted across major cities in Ukraine, Kazakhstan and Azerbaijan from the first half of 2013 until the end of 2015 have showed that prevalence of COPD diagnosed by spirometry (based on the Global Initiative for Chronic Obstructive Lung Disease guideline (2011) (FEV1/FVC < 0.70) was 31.9% (95% CI 21.7–45.3) per 1000 of population in Ukraine. Remarkably, almost all (90%) of the deaths caused by COPD occurred in low and middle-income countries (LMICs). Bangladesh, like Ukraine and other LMICs, is facing epidemiological transition with an increasing burden of non-communicable diseases like COPD. Currently, 12.5% of Bangladeshi adults are suffering from COPD. However, there are conflicting data suggesting that COPD is a more prevalent cause of death in the more affluent regions: while COPD-associated mortality rate was 42.6 and 50.4 deaths per 100,000 populations in high- and medium-income countries, respectively, it was only 16.7 in low-income countries. As reported in BOLD (Burden of Obstructive Lung Disease) study, lack of correlation between the prevalence of smoking and COPD-associated mortality can be attributed to a reverse relationship between poverty and the prevalence of smoking.

An important consideration to take into account when managing the process of shaping the models of prevention, diagnosis and treatment of COPD includes complications and comorbidities. COPD is known to be associated with quite a number of comorbid conditions. As reported in the CORE study, comorbidities were documented in 44.2% of COPD patients in Ukraine, 23.5% of COPD patients in Kazakhstan and 54.6% of COPD patients in Azerbaijan. The main target organs in COPD include the bronchi and the lungs; however, other organs and systems are also affected at certain stages of the disease. As expected, the most significant correlation was found between COPD and cardiovascular diseases (CVDs). COPD was found to be a predecessor of CVDs, which surpass other diseases as the leading cause of death in the world, accounting for 45.1% of mortality, with the majority of it due to coronary heart disease. Arterial hypertension (AH) is part of the group of CVDs that symbolize the highest proportion of diseases mortality causes such as cerebral vascular accident and acute myocardial infarction, reaching about two-fifths of the adult population in developed countries. Hong-lei Yin et al. have found that the incidence of arterial hypertension was also significantly higher in patients with COPD.

Both AH and COPD are genetically determined conditions with multiple genes, combinations of genes, inter-gene interactions and epigenetic processes responsible for their occurrence. For example, inflammatory biomarkers, including cytokines, are associated with both the lung disease and the systemic pathology. Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are increased in COPD, and appear to amplify inflammation, thereby leading to the increased expression of multiple inflammatory genes. However, no data for association between gene polymorphism and arterial hypertension in patients with COPD in Ukraine have ever been internationally published.

Objective: to establish the role of insertion-deletion polymorphism of the angiotensin-converting enzyme gene in development of arterial hypertension in patients with chronic obstructive pulmonary disease.

Research Methods

Patients. The study group consisted of 96 patients admitted to the Ternopil University Hospital. We stratified patients in three groups: Group 1 (25 patients with COPD), Group 2 (23 patients with arterial hypertension [AH]), Group 3 (28 patients with COPD + AH). The control group consisted of the 20 healthy subjects. As inclusion criteria, were selected: sex of the patient (males), age of the patient (40 to 60 years), an established diagnosis of COPD or/and AH.
As for the exclusion criteria, it was excluded: bronchial asthma, α1-antitrypsin deficiency, active tuberculosis, lung cancer, significant bronchiectasis, sarcoidosis, pulmonary fibrosis, interstitial lung disease; signs and symptoms of clinically significant neurological, psychiatric, renal, hepatic, immunological, gastrointestinal and urogenital disorders, musculoskeletal conditions, disorders of the skin and sensory organs, endocrine disorders (uncontrolled diabetes or thyroid disease) or uncontrolled hematological disease, unstable liver disease, unstable or life-threatening heart disease, patients with malignancies who have not been completely disease-free for a minimum of 5 years, drug and alcohol abuse.

COPD was diagnosed according to the guidelines published by American Thoracic Society and European Respiratory Society (GOLD, 2013). Airway obstruction was assessed using GOLD classification, 2008. The diagnosis of COPD with moderate (Stage 2) airway obstruction was confirmed with compatible clinical features concurrent with airflow limitation defined as forced expiratory volume in 1 second (FEV1) / forced vital capacity (FVC) less than 0.70 (FEV1/FVC ratio of 50–79% predicted).

The diagnosis of arterial hypertension (Stage I) was made according to 2018 ESC/ESH Guidelines for the management of arterial hypertension. Systolic (140-159 mmHg) and/or diastolic (90-99 mmHg) blood pressure were considered as the presence of Stage I AH. Left ventricular hypertrophy was confirmed by an electrocardiogram.

**Analytical Methods.** Blood, in a fasting state, was drawn by venous puncture. Plasma ACE activity was determined photometrically by a commercially available kinetic kit purchased from Sigma Aldrich (USA). Testing was performed according to the manufacturer’s instructions. The assay is based on the cleavage of a synthetic fluorogenic peptide. The measured fluorescence is directly proportional to the ACE activity present. The measurement was automatically performed by COBAS INTEGRA 400 Plus Analyzer (Roche Diagnostics, Switzerland).

Venous blood for genotyping was sampled under sterile conditions into 2.7 mL Monovettes containing potassium salt of ethylenediaminetetraacetic acid (EDTA) as an anticoagulant; the samples were frozen and stored at –20 °C. Molecular genetic studies included extraction of DNA and use of polymerase chain reaction with subsequent analysis for the length of restriction fragments. In brief, a set of primers was designed to encompass the polymorphic region in intron 16 of the ACE gene (sense primer 5′ CTGGAGACCCACTCCCCATCTTTTCT 3′ and antisense primer 5′ GATGTGGCCATCACATTGTCGAT 3′). The polymerase chain reaction (PCR) reaction contained 100 ng DNA template, 0.125 μmol/L of each primer, 200μmol/L 4dNTPs, 1 unit of Taq DNA polymerase, and 1.5 mmol/L MgCl₂ and 5% dimethyl sulfoxide. DNA was amplified for 30 cycles, each cycle composed of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute, with a final extension time of 3 minutes. Amplification products yielded were of 439 bp for the D allele and 727 bp for the I allele. The PCR products were separated by electrophoresis on 2% agarose gel and identified by ethidium bromide staining.

**Statistical Analysis.** Data were analyzed by the STATISTICA 10.0 software. Normality of data was analyzed using the Kolmogorov-Smirnov test. Non-Gaussian variables were reported as medians and interquartile range (IQ, percentile 25 and percentile 75). Quantitative variables were compared with Mann-Whitney U test. P values of 0.05 or less were considered statistically significant. Pearson correlation analysis and point-biserial correlation analysis were performed to investigate the correlations between variables. P values of 0.05 or less were considered statistically significant. Assessment of genotypes of the selected sample for conformity to general population sample was guided by the Hardy-Weinberg principle. The observed frequencies and the expected frequencies calculated from the following expression: p² + 2pq + q² = 1 (Hardy-Weinberg equilibrium) were compared using Pearson chi-square, χ². In case of probability value p>0.05, a null hypothesis of equal samples was accepted, i.e. that the selected sample was equivalent to the general population.

**Ethical approval:** The study protocol was approved by the Medical Ethics Committees of I. Horbachevsky Ternopil State Medical University (No 55-04/11/2019) and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983. Informed consent was obtained from all patients.
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**Results**
The analysis of parameters in the main groups of patients with COPD has shown that the age of patients with concomitant AH was significantly higher (by 20.52%) compared with patients with COPD alone. Body weights of Stage 2 COPD patients with comorbid Stage I AH were significantly higher compared to COPD-only patients with no comorbidities. The mean disease duration was found to range within 5 to 6 years in COPD-only patients with no comorbidities. In males with comorbid Stage 2 COPD and Stage I AH, disease duration was almost twice longer compared to patients in other groups (Table 1). These data do not have a uniform explanation and call for further analysis.

**Table 1. Characterization of patients with chronic obstructive pulmonary disease depending on their comorbidities**

| Characteristics            | Group 1 (COPD) | Group 3 (COPD+AH) |
|----------------------------|----------------|-------------------|
| Age, years                 | 45.96±2.50     | 55.39±1.97*       |
| Body weight, kg            | 80.48±2.82     | 94.54±3.29*       |
| Height, cm                 | 173.68±1.34    | 169.39±6.03       |
| HR, bpm                    | 76.68±2.34     | 81.21±2.26        |
| sBP, mmHg                  | 131.60±2.23    | 147.68±2.54*      |
| dBP, mmHg                  | 82.80±1.50     | 93.39±1.34*       |
| RR, respirations/min       | 21.56±0.38     | 21.68±0.26        |
| Disease duration, years    | 5.13±0.98      | 11.68±1.38*       |

Notes: * - there is a significant difference between the findings in COPD patients with and without concomitant AH.
Recent studies have shown human ACE levels to be genetically determined. The ACE gene is located in chromosome 17, on 17q23 locus. The polymorphism of this gene involves the presence (I, insertion) or the absence (D, deletion) of 287 base pairs of the Alu repeat in intron 16 of the ACE gene. The 3 respective genotypes include insertion homozygotes (II), deletion homozygotes (DD) and heterozygotes (ID)\(^{29}\). The frequency distribution of polymorphic genotypes of the gene encoding ACE and assessment of compliance with the Hardy-Weinberg population equilibrium were carried out in groups of patients with COPD, AH and with COPD+AH combination. The frequencies of the genotype responsible for I/D polymorphism of the ACE gene in the control and experimental groups were not found to deviate significantly from the Hardy-Weinberg equilibrium (p>0.05) (Table 2).

**Table 2. Hardy-Weinberg equilibrium of the ACE gene I/D polymorphism in COPD, AH and their combination**

| Genotype                  | COPD    | AH      | COPD+AH | Control |
|---------------------------|---------|---------|---------|---------|
|                           | Expected| Observed| Expected| Observed| Expected| Observed| Expected| Observed|
| Common homozygotes        | I/I     | 7.8     | 7.3     | 7       | 9       | 6.1     | 5       |
| Heterozygotes             | I/D     | 14.6    | 11.3    | 12.5    | 12.9    | 9.9     | 12      |
| Rare homozygotes          | D/D     | 2.6     | 4       | 4.4     | 6.1     | 7       | 4       |

Notes: COPD - chronic obstructive pulmonary disease; AH – arterial hypertension.

The frequencies of alleles for the ACE gene in patients with COPD, AH, COPD + AH and control group patients are shown in Table 3. In the COPD group, the established distribution was ACE I allele (56.0 %) and ACE D allele (44.0 %); in the AH group - 56.5 % and 43.5 %; in the COPD+AH group - 53.6 % and 46.4 %. However, these data significantly did not differ from the control group.

**Table 3. Allele frequencies of the ACE gene I/D polymorphism**

| Allele Frequency | COPD | AH | COPD+AH | Control |
|------------------|------|----|---------|---------|
|                  | n    | %  | n       | %       |
| ACE I allele     | 28   | 56.0 | 26       | 56.5    | 30       | 53.6    | 22       | 55.0    |
| ACE D allele     | 22   | 44.0 | 20       | 43.5    | 26       | 46.4    | 18       | 45.0    |

Notes: COPD - chronic obstructive pulmonary disease; AH – arterial hypertension.

Compared to controls, the activity of angiotensin-converting enzyme was significantly higher in the group of patients with AH (by 58.66%) and in the group of patients with COPD and AH (by 64.84%) (Table 4). The results obtained are apparently even more significant when ACE activity is assessed depending on genotype carrier status (in relation to the ACE gene). In patients with II genotype, the highest ACE activity was seen in test groups 2 and 3; however, ACE activity in these patients was the lowest compared to other genotypes. In patients with ID genotype, ACE activity in test groups 2 and 3 was also superior to both controls and the group of patients...
diagnosed with COPD. Statistically, ACE activity in ID-genotypic patients with AH/AH + COPD was significantly higher than in II-genotypic patients; however, it was still significantly lower compared to DD-genotypic patients. Analysis of ACE activity in DD-genotypic patients suggests its highest values in test Group 3, a significant superiority over controls (a 65.63% exceedance), over Group 1 (a 63.08% exceedance) and over Group 2 (a 7.07% exceedance) (Table 4). The results obtained suggest that the absolute highest ACE activity was seen in combined COPD+AH patients irrespective of their ACE gene genotype. However, it should be noted that comparison of genotype-dependent ACE activity has demonstrated ACE activity to be the highest in patients with DD genotype, which was true for both controls and subjects in test groups.

*Table 4. Plasma activity of angiotensin-converting enzyme in patients with COPD and hypertension depending on ACE genotype*

| Genotype | Controls, n=20 | Group 1 (COPD), n=25 | Group 2 (AH), n=23 | Group 3 (COPD+AH), n=28 |
|----------|----------------|----------------------|--------------------|------------------------|
| II       | 5.40 [5.20; 5.60] | 5.50 [5.30; 5.70] | 8.70 [8.40; 9.20] | 8.90 [8.60; 9.30] |
| ID       | 5.90 [5.70; 6.10] | 5.90 [5.80; 6.10] | 9.50 [9.00; 10.00] | 9.60 [9.20; 9.80] |
| DD       | 6.40 [6.30; 6.60] | 6.50 [6.40; 6.70] | 9.90 [9.50; 10.30] | 10.60 [10.20; 11.00] |

Total ACE activity

5.83 [5.50; 6.13] | 5.88 [5.60; 6.10] | 9.25 [8.65; 9.75] | 9.61 [9.05; 10.13] |

Note 1. * - the intragroup difference between overall ACE activity and carrier status of I and D genotypes of the ACE gene is significant (p<0.05–0.001).
Note 2. # - the difference of overall ACE activity vs. controls is significant (p<0.05–0.001).
Note 3. p< is the significance of differences between II genotype and ID genotype.
Note 4. p< is the significance of differences between ID genotype and DD genotype.
Note 5. p< is the significance of differences between II genotype and DD genotype.
Note 6. p< is the significance of differences between controls and test groups within the II genotype.
Note 7. p< is the significance of differences between controls and test groups within the ID genotype.
Note 8. p< is the significance of differences between controls and test groups within the DD genotype.
Note 9. p< is the significance of differences between Group 3 and other test groups within one genotype.

In order to detect statistical significance of relationships between ACE genotype and selected clinical and hemodynamic characteristics of hypertension in patients with COPD, we have performed a correlation analysis of these parameters using a χ2-method. Patients with COPD and AH were found to have a direct, robust and significant relationship of all ACE genotypes with systolic/diastolic blood pressure. The II genotype had a positive relationship with patient age and a negative relationship with body weights and respiratory rates of COPD + AH patients. The ID genotype was associated with faster respiratory rates; however, its correlation with disease duration was negative (Table 5).

*Table 5. Correlations between selected clinical and hemodynamic characteristics of patients with chronic obstructive pulmonary disease and hypertension and ACE genotypes*

In order to detect statistical significance of relationships between ACE genotype and selected clinical and hemodynamic characteristics of hypertension in patients with COPD, we have performed a correlation analysis of these parameters using a χ2-method. Patients with COPD and AH were found to have a direct, robust and significant relationship of all ACE genotypes with systolic/diastolic blood pressure. The II genotype had a positive relationship with patient age and a negative relationship with body weights and respiratory rates of COPD + AH patients. The ID genotype was associated with faster respiratory rates; however, its correlation with disease duration was negative (Table 5).

Note: * = the probability factor is statistically significant.

Discussion

COPD is frequently accompanied by various comorbidities. Such comorbidities as pulmonary arterial disease and nutrient deficiencies are directly induced by COPD while others, such as systemic venous embolism, anxiety, depression, osteoporosis, obesity, metabolic syndrome, diabetes, pancreatitis, sleep disturbances and anemia lack any apparent pathophysiological connections to COPD. Chronic systemic inflammation is a common mechanism behind most of those extrapulmonary manifestations. A cross-sectional study using data from KNHANES 2010–2012 suggests that only such comorbidities as arterial hypertension and lung tuberculosis in patient’s life history were independently associated with COPD (adjusted for age, smoking status and other risk factors). Our preliminary results suggest a high incidence of COPD combined with AH, since among 143 patients with Stage 2 COPD 28.67% had no comorbidities; in 27.27% of patients, the course of their underlying disease was accompanied by Stage I AH and in 43.36% of patients COPD was associated with other comorbidities. Analysis of clinical findings in patients with COPD + AH has demonstrated higher values of body weight and respiratory rates of COPD + AH patients.
and systolic/diastolic blood pressure in this category of patients; concerning data in Group 1, the age and duration of the disease were also significantly higher. Studies by other authors have shown that the development of COPD and AH is influenced by such risk factors as age and smoking. In the meantime, a significant relationship was found to exist between COPD and AH, which did not depend on the duration of either disease.44

Analysis of literature data suggests that patients with COPD and low body mass index are at high risk for bronchial obstruction, osteoporosis, abdominal aorta aneurysm, peripheral vascular disease and substance abuse.45 On the other hand, people with COPD and high body mass index are at lower risk for bronchial obstruction and osteoporosis; however, they were found to have a number of comorbidities and cardiovascular risk factors with increased systemic inflammation.46 Divo M.J. et al. suggest that adipose tissue is playing a modulating role in development and progression of cardiovascular disease in patients with COPD.47 Numerous studies have demonstrated that patients with excessive weight/obesity and COPD are at lower risk for all-cause mortality.48,49 This phenomenon is known as obesity paradox, defined as a reverse causality between survival and obesity seen in various chronic disease.

Autonomous dysfunction is a probable mechanism linking COPD and AH. COPD-associated airway obstruction develops as a result of progressive airway inflammation, leading to parenchymatous destruction, swelling of mucus membranes, airway remodeling, mucoid impaction and increase in cholinergic tone of smooth muscles in the airways.40 Such factors as recurrent hypoxemia and hypercapnia, increased intrathoracic pressure due to airway obstruction and chronic airway inflammation found in COPD overstrain sympathetic nerves and reduce the sensitivity of baroreceptors. On the other hand, some studies prove that development, progression and outcomes of human hypertension are associated with impaired autonomous control of the cardiovascular system, especially when sympathetic section is subject to abnormal activation.41,42

The relationship between the polymorphism of the gene encoding ACE and COPD has been addressed in numerous prior studies; however, these studies have yielded ambivalent results.43-45 Prior studies have demonstrated the role of genetic factors in susceptibility to COPD; in part, susceptibility to COPD was found to be associated with polymorphism of proteinase-activated receptor-1,46 plasminogen activator inhibitor-1,47 and β2-adrenergic receptors.58 A study of relationship between COPD and ACE activity has shown the activity of ACE to depend on oxygen concentrations in the blood; therefore, increases in ACE levels due to COPD-associated hypoxia may cause severe tissue damage.59

ACE is an endopeptidase consisting of two catalytic domains; this enzyme is usually expressed by endothelial, epithelial and neuronal cells.50 It exists both in a membrane-bound form (ACE) and a soluble form (sACE), the latter produced through exposure to zinc metalloprotease (referred to as “ACE secretase”), which cleaves the mature membrane-bound ACE in the juxtamembrane extracellular domain to release the large extracellular part of the enzyme.51 The known function of ACE is associated with the renin-angiotensin system, wherein ACE catalyzes the synthesis of angiotensin II vasoconstrictor from its non-vasoactive precursor, angiotensin I, and is also responsible for inactivation of vasodilator bradykinin.52 Angiotensin II is a powerful vasopressor, which regulates blood pressure and fluid and electrolyte balance, chiefly through biosynthesis of aldosterone.53-54

Association between I/D polymorphism of the ACE gene and the risk for COPD is a very timely and widely studied issue; however, existing data need to be clarified and appended.45-57 The low activity of ACE was recognized to play a positive role in the development of COPD.58 It is worth noting that the DD genotype of ACE is generally associated with increased circulating and cellular levels of ACE and increased cardiovascular risk.59 However, most of overall ACE activity in the body is located in the pulmonary tissue and its activity is additionally increased in chronic hypoxia, which develops in COPD. Therefore, ACE in the pulmonary tissue may be involved in the pathogenesis of pulmonary hypertension secondary to COPD.

A few studies show insertion/deletion (I/D) polymorphism in the ACE gene to be related to increases in plasma ACE levels.60 Analysis of other published studies on whether ACE gene insertion/deletion polymorphism was associated with the risk of chronic obstructive pulmonary disease suggests lack of significant association between I/D polymorphism and COPD. Carriers of II and ID genotypes had significantly lower levels of circulating angiotensin-converting enzyme than subjects with DD genotype.58 A noteworthy detail is that an interaction between allele 894G of the gene encoding endothelial nitrogen oxide synthase and
allele I of the ACE gene may reduce vasoconstriction and increase vasodilatation, which is a positive effect in both COPD and hypertension. In our study, greater proportions of II homozygotes were seen both among patients with AH and among patients with combined COPD + AH; however, no significant relationship was found between the I allele and the incidence of conditions explored in this study. In the meantime, II genotype was associated with lower respiratory rates, which may be an indirect effect of vasodilatation.

Conclusions
We did not observe a significant correlation between I/D polymorphism in the ACE gene and the risk of arterial hypertension in COPD patients; therefore, comprehensive studies on Ukrainian patients are required. The highest activity of ACE was found in patients with combination of COPD and arterial hypertension; maximum values of ACE activity were seen in patients with DD genotype.
The II genotype had a positive relationship with patient age and a negative relationship with body weights and respiratory rates of COPD and AH patients. The ID genotype was associated with increased respiratory rates; however, its correlation with the duration of the disease was negative.

Study limitation. Our current study with small sample size cannot answer all questions about the association between COPD and AH, but it is the starting point for future multi-center investigations in Ukraine with large population which will determine the various aspects of the correlation between COPD and arterial hypertension.

Conflict of interest
All the authors declare no conflict of interest

Authors’ Contribution:
Idea owner of this study: Marushchak M.
Study design: Marushchak M., Maksiv K.
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