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EVALUATION OF THIOL COMPOUNDS AND LIPID PEROXIDATIVE PRODUCTS IN PLASMA OF PATIENTS WITH COPD

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ABSTRACT
Cigarette smoking is the major etiological factor in the chronic obstructive pulmonary disease (COPD) and the local inflammation and oxidative stress are considered as primary pathogentic mechanisms of COPD. The aim of the current preliminary study was to adapt for microanalyses methods for assessment of the products of lipid peroxidation (MDA) and thiol compounds in plasma, to investigate the levels of those biological markers of oxidative stress and antioxidant defense in patients with COPD and to determine factors affecting those indexes. Methods: MDA and thiol compounds were evaluated in 14 patients with COPD and in 12 non-affected control individuals.
Results: The values of MDA were significantly higher ($p<0.0001$) (Figure 3A) and of the thiols were significantly lower ($p=0.048$) (Figure 3B) in patients with COPD compared to those in controls. The levels of MDA were significantly higher in patients who had smoked or were current smokers (4,800±0,932 mmol/l) than non-smokers (3,351±0,635 mmol/l, p=0.0.28). We found a strong significant inverse correlation between MDA levels EFV1% pr. ($R= -0.500$, $p=0.069$), however there was no correlation with other characteristics of lung function (FEV1/FVC % и PEF). In addition, there was a significant positive correlation of the BMI with MDA levels ($R=0.482$, $p=0.020$) and significant inverse correlation with thiol levels ($R= -0.470$, $p=0.024$) in plasma of all investigated individuals (both controls and patients with COPD).
In conclusions based on the results of our preliminary study we suggest the presence of systemic oxidative stress in COPD, which is severe in more advanced stage and confirm the role of smoking in its aggravation. The overweight might contribute to the deterioration of the imbalance of oxidants/antioxidants both in COPD patients and unaffected individuals

Key words: COPD, thiols, MDA, smoking, BMI

INTRODUCTION

The chronic obstructive pulmonary disease (COPD) is a slow, progressive condition characterized by airflow limitation, which is largely irreversible (1).

The restriction of airflow is a result from the inflammation in airways triggered by inhaled noxious gases and particles. Cigarette smoking is the major etiological factor in this condition and the local inflammation and oxidative stress are considered as primary pathogentic mechanisms of COPD. An increased oxidant burden in smokers derives from the fact that cigarette smoke enormous quantity of free radicals (ROS, RNS etc.) and chemicals: more than 1014 oxidant/free radicals per puff and more than 4700 chemicals (2, 3). The short living ROS, such as superoxide radical ($O_2^{\cdot-}$) and nitrogen oxide (NO), which are mainly in the gas phase of cigarette smoke, can react spontaneously forming the highly toxic oxidizing peroxynitrite anion ($ONOO^-$). The solid (tar) phase also contains variety of free radicals with organic nature (such as long lasting semiquinone radicals), which react with $O_2^{\cdot-}$ and form hydroxyl radical ($OH^{\cdot}$) and H_2O_2 (4). The oxidant burden in the lungs is further enhanced in smokers by the release f ROS

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from alveolar macrophages and sequestered neutrophils in the lung (5).

Thus the presence of a strong inflammation process in the wall of airways in COPD, which is determined by the great number of activated neutrophils and macrophages and increased amount of inflammatory mediators, significantly contributes to the imbalance between oxidants and antioxidants in favour of oxidants, i.e. contributes to the aggravation of the oxidative stress, which is considered for an important factor in pathogenesis of COPD (6).

The performed investigations with patients with COPD have unequivocally showed that there is an oxidative stress in the lung, whereas the occurrence of systemic oxidative stress and the correlations between the indexes of the systemic oxidative stress and severity of the disease are still debatable and the results are conflicting (4, 7-16, 17, Ceylan, 2006 #328).

In this respect the aim of the current preliminary study was to adapt for microanalyses methods for assessment of the products of lipid peroxidation (MDA) and thiol compounds in plasma, to investigate the levels of those biological markers of oxidative stress and antioxidant defense in patients with COPD and to determine factors affecting those indexes.

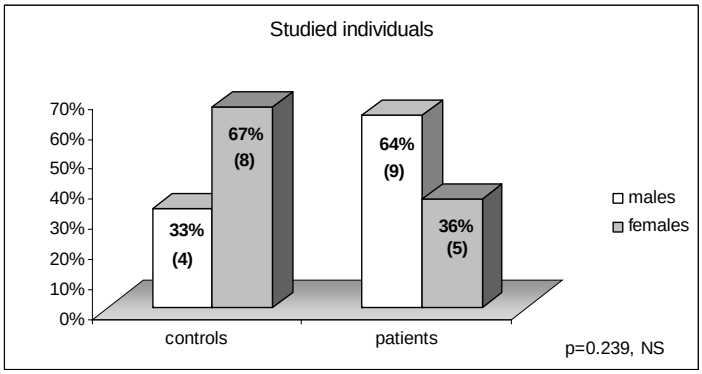
MATERIAL AND METHODS

Patients and controls

In the current preliminary study there were enrolled 14 patients with COPD with mean age of 63.36±6 years (57-74 years). The clinical stages of COPD according to the GOLD (Global Initiative for Chronic Obstructive Lung Disease, 2009) (1) were as the following: 5 patients suffered from moderate COPD (GOLD II) and 9 from severe COPD (GOLD III). The inclusion criteria for COPD were as the following: age higher than 40 years; forced expiratory volume in one second (FEV1) of <80%; forced expiratory volume in one second (FEV1)/ forced vital capacity (FVC) ratio of ≤ 70%; FEV1 reversibility after inhalation of 400 μg Salbutamol of <12%.

The control group consisted of 12 healthy voluntaries aged between 23 and 79 years (mean of 47.42±15 years). The distribution of controls and patients according to the gender and smoking habit is presented in **Figure 1A and 1B**, respectively.

A



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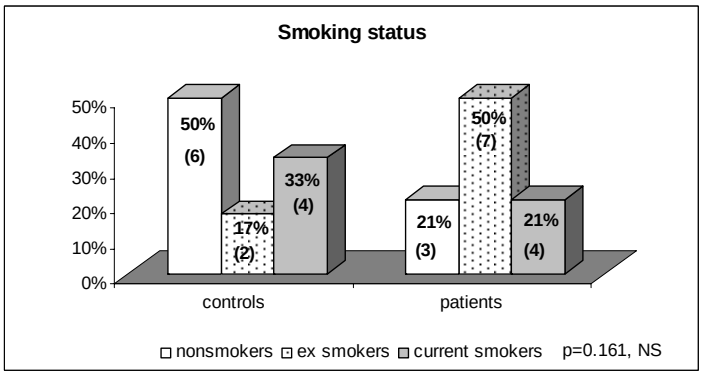

Figure 1. The distribution of controls and patients according to the gender (A) and smoking habit (B).

Laboratory methods

Assessment of the lipid peroxidation products

The lipid peroxidation products, express also as thiobarbituric-reactive substances, (TBARs) or malone dialdehyde (MDA) have been accepted as a reliable index for the presence of oxidative stress. They are products from the ROS-generated peroxidative damage of unsaturated fatty acids in the phospholipids in cell membrane and in lipoprotein complexes in plasma.

We adapted for microanalysis the procedure reported by Pasha and Sadasivadu (18-20) which is presented in **Figure 2.**

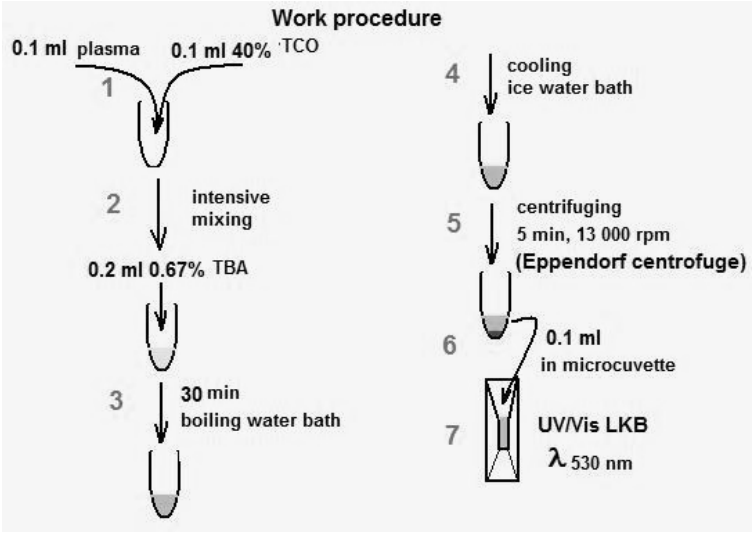

Figure 2. The work procedure adapted for microanalysis of MDA.

The calculations were performed according to the following formulas:

C [mol/l] = 4xOD/kL

where:

k = Molar extinction coefficient (1.5×10^5 $M^{-1}cm^{-1}$)

OD = Extinction/Absorbance/ Optical density

C = Concentration (mol/l)

L = Length of the beam/ width of cuvette used (1 cm)

4 – Coefficient for recalculation of the concentration in plasma according to the dilution in reaction mixture.

Assessment of thiol compound

The main thiols in plasma are the reduced glutathione (GSH), the amino acid cysteine and in less extend homocysteine and the plasma proteins. However most f the proteins are precipitated by methanol during the work procedure and discarded, which results in detection by the adapted method mainly of the low molecular weight thiols. Thiol compound in plasma have a significant effect for redox state of plasma and they are important antioxidants.

The assessment of thiols in plasma was performed by the adapted for microanalysis method of Ellman (21, 22).

The method is based on the interaction of reduced thiols (i.e. glutathione) with the reagent of Ellman [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB). The reduction of Ellman's reagent by the thiol group leads to the 2-nitromercaptobenzoic acid anion, which has an intensive yellow color and can be measured spectrophotometrically at 412 nm. In brief: in an eppendorf tube with 100 µl plasma it was added 150 µl 0,2 M Tris-HCl, pH 8.2, containing 0.02 M EDTA, 10 µl 10 mM DTNB in methanol and 740 µl methanol to a final volume of 1 ml (final concentrations of 0.1 mM DTNB and 0.03 M Tris-HCl, pH 8.2). After incubation at 25^0C for 15 min the reaction mixture was centrifuged at 13400 rpm and the precipitated protein pellet was removed. The optical density of the yellow product was measured at 412 nm against a blank probe. Aqueous solutions of known GSH concentration, in range 0.25 to 2 mM GSH (SERVA Electrophoresis GmbH, Germany) were used for creating of the standard curve for each series of measurements. The results were expressed in mmol/l (mM) GSH.

Statistical analyses

Statistical analyses were performed using StatView v.4.53. for Windows (Abacus

Concepts, Inc.). The ANOVA test was applied for comparing the continuous variables in independent groups. The frequencies of distribution in contingency tables were analyzed using Chi2 test. The relationship between two different continuous parameters was analyzed by the Pearson correlation test. Factors with $p<0.05$ were considered statistically significant.

RESULTS

The values of MDA were significantly higher ($p<0.0001$) **(Figure 3A)** and of the thiols were significantly lower ($p=0.048$) **(Figure 3B)** in patients with COPD compared to those in controls.

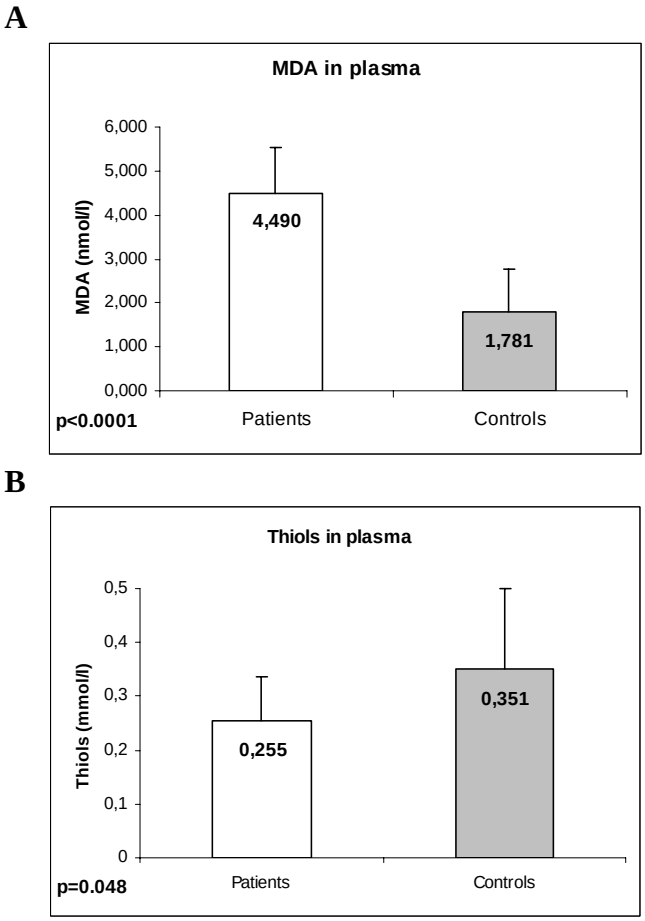

Figure 3. Comparison of the values of MDA (A) and thiols (B) in plasma of patients with COPD and control individuals.

We found a tendency for inverse relationship between the levels of MDA and thiol compounds in plasma of all investigated individuals (both controls and patients) ($R= -0.336$, $p=0.094$) **(Figure 4A)**. The same tendency was seen in the group of controls ($R= -0.367$, $p=0.229$) **(Figure 4B)**, whereas, paradoxically, in the group of patients there was a tendency for positive correlation between those values ($R=0.472$, $p=0.088$) **(Figure 4C)**.

The latter observed tendency is difficult to be explained. There are reports describing that malondialdehyde, which is a typical end-product from peroxidized polyunsaturated fatty acids, is highly reactive and binds the biomolecules such as proteins and polynucleotides (23). The major reactive site of proteins with MDA is the ε-amino group of lysine residues, resulting in forming cross-links in the proteins and leading to their chemical denaturation by elimination of the positive charge of lysine (24, 25). Hence, we could assume that in the presence of lower levels of reduced thiols, MDA and the other aldehydes generated from the lipid peroxidation could more easily bind and modify lysine moieties in the proteins that finally might result in lowering of MDA levels.

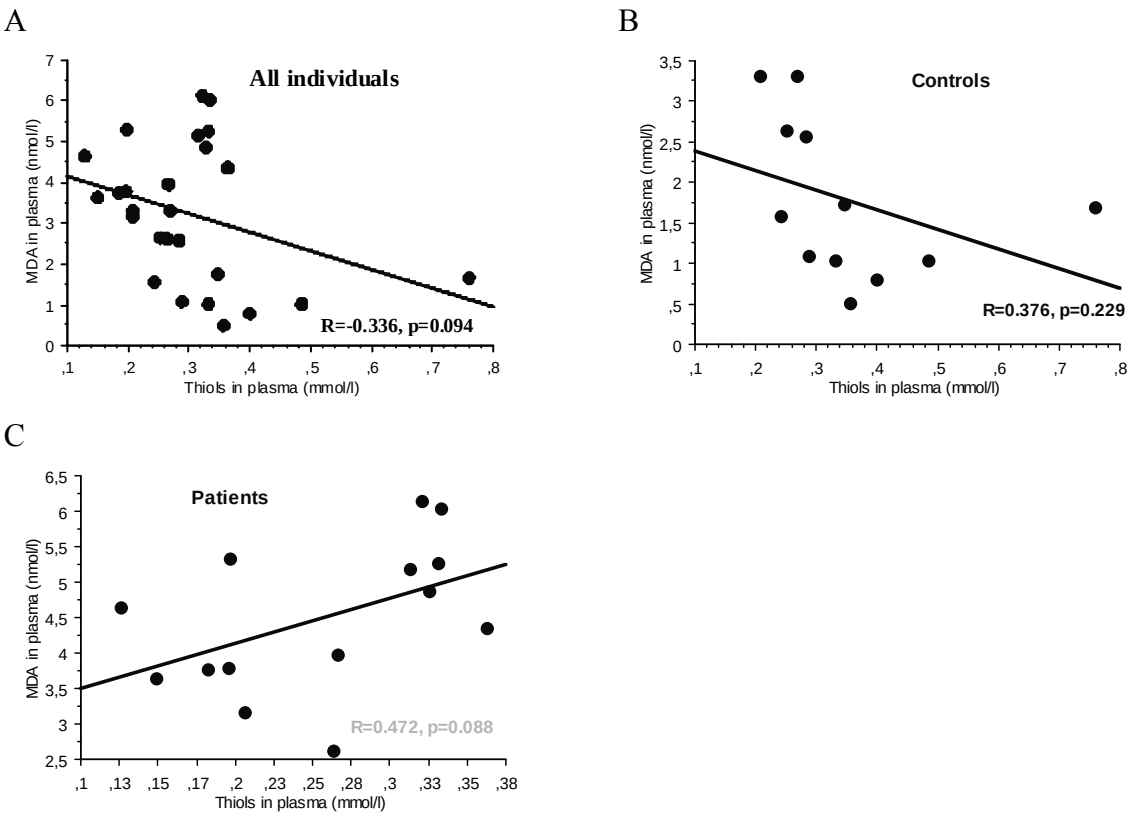

Figure 4. Correlations between MDA and thiol levels in plasma of all studied individuals (A), in controls (B) and in patients with COPD (C) separately.

We also found that the levels of MDA were significantly different between patients who had smoked or were current smokers and those who had never smoked: smokers had significantly higher MDA levels in plasma (4,800±0,932 mmol/l) than non-smokers (3,351±0,635 mmol/l, p=0.0.28) **(Figure 5)**. This tendency existed also in controls, but did not reach statistical significance **(Figure 5).**

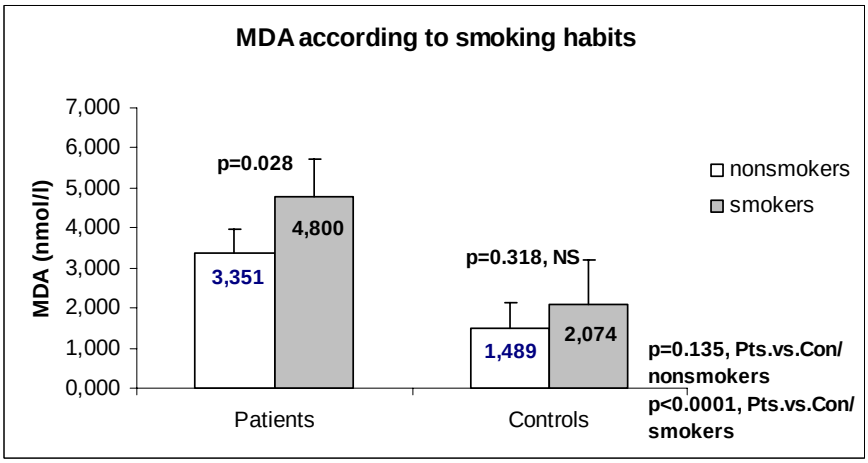

Figure 5. Comparison of the MDA levels in plasma of patients and controls according to the smoking habits.

Surprisingly the levels of thiols in plasma showed similar tendency, but there were no statistical significant difference either in the group of patients or in controls **(Figure 6)**.

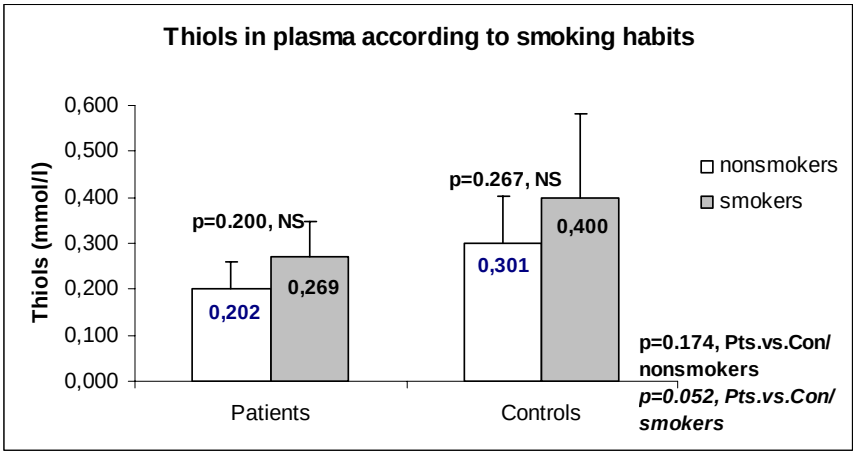

Figure 6. Comparison of the thiol levels in plasma of patients and controls according to the smoking habits

Interesting associations were observed between the studied indexes of oxidative stress and antioxidant defense and the body weight. We found a significant positive correlation between the BMI and MDA levels in plasma of all investigated individuals (both controls and patients with COPD) (R=0.482, p=0.020) **(Figure 7A)**. This correlation was even stronger in females (R=0.886, p=0.0006) **(Figure 7B)**. The statistical significance remained in the subgroup of female patients (R=0.865, p=0.026), whereas in controls there was only tendency (R=0.691, p=0.197). In males there was similar association, but markedly less evident (R=0.123, p=0.682).

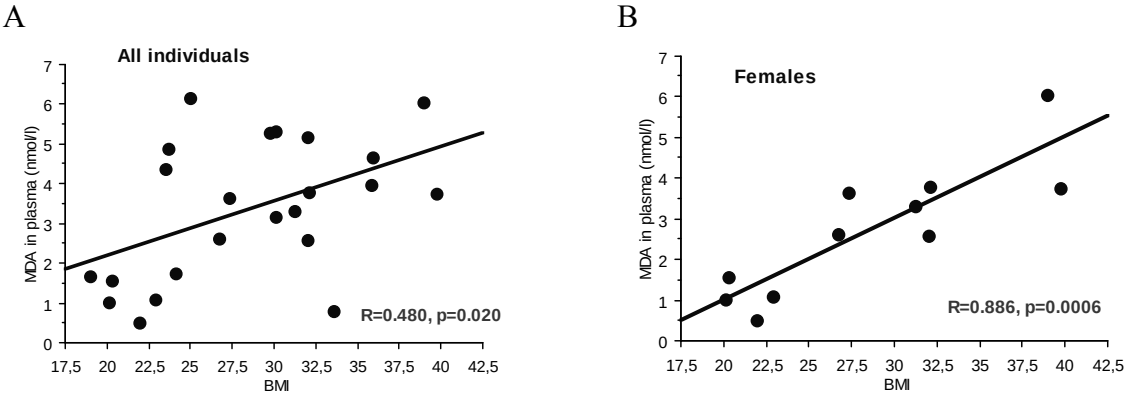

Figure 7. Correlations between plasma MDA levels and BMI in all investigated individuals (A) and in females only (B)

An opposite, the plasma thiols' levels showed significant inverse correlation with BMI of all investigated individuals ((R= -0.470, p=0.024) **(Figue 8A).** This correlation was more prominent in males (R= -0.684, p=0.014) **(Figure 8B)**, although it existed also in females (R= -0.317, p=0.345). The observed correlation remained significant in the subgroup of male patiernts (R=-0.677, p=0.045), whereas in controls t was not significant (R= -0.690, p=0.516).

One important goal of our study was to evaluate if the analyzed plasma indexes (MDA and thiols' levels) are associated with the severity of the disease and the lung function. In this respect we compared the plasma levels of these oxidative state characteristics in patients with different GOLD COPD stages. It has appeared that patients with moderate COPD (stage II) had lower levels of MDA (3.969±0.666 mmol/l) compared to that with severe COPD (stage III, 4.779±1.149), although this difference was not significant (p=0.178) **(Figure 9)**. The thiols' plasma levels were commensurable in patients with moderate and severe COPD (p=0.875) and did not correlate with the spirometric characteristics of lung function.

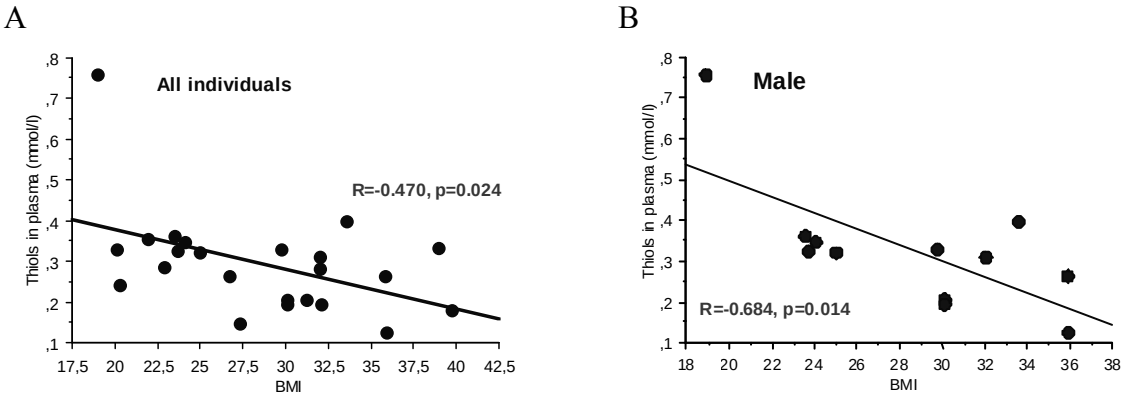

Figure 8. Correlations between plasma levels of thiol compounds and BMI in all investigated individuals (A) and in females only (B)

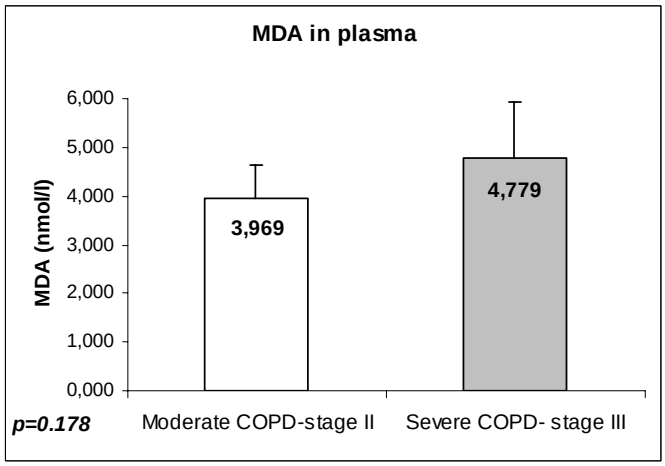

Figure 9. Comparison of MDA levels in plasma of patients with moderate (GOLD II) and severe (GOLD III) COPD.

When the MDA levels of patients with COPD were analyzed for association with the spirometric characteristics, we found a strong significant inverse correlation between MDA index of oxidative stress and EFV1% pr. (R= -0.500, p=0.069) **(Figure 10)**, however there was no correlation with other characteristics of lung function (FEV1/FVC % и PEF).

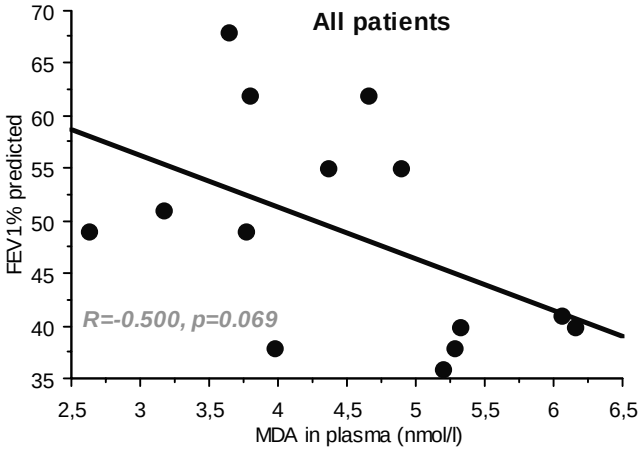

Figure 10. Correlation between the MDA plasma levels and FEV1 % pr. Of patients with COPD.

DISCUSSION AND CONCLUSIONS

The results of our preliminary study give us a ground to conclude that there is a systemic oxidative stress in patients with COPD, which is more prominent in more severe stage of the disease. This conclusion is additionally supported by the observation that MDA levels inversely correlated with one of the main spirometric characteristics for the lung function (FEV1% predicted). These findings of ours coincide with those previous once indicating the presence of systemic oxidative stress (10, 26, 27).

Our finding that MDA levels were higher in patients with COPD who were ex- or current smokers in comparison to that who had never smoked, suggest that smoking significantly contributes to aggravation of the oxidative stress. Similar results were reported for healthy smokers and for patients with smoking-associated COPD in comparison to healthy non-smokers (26-29)

Interesting finding of our study is that the MDA levels inversely correlated with BMI, whereas the antioxidant thiols' levels had a positive relationship with BMI. These findings correspond to several other studies that have explored the association between the body weight or obesity and oxidative stress indexes and characteristics in different individuals' group and conditions (30-33) and confirm the suggestion that overweight and obesity may induce oxidative stress and decreased on antioxidant capacity of plasma.

In conclusions based on the results of our preliminary study we suggest the presence of systemic oxidative stress in COPD, which is severe in more advanced stage and confirm the role of smoking in its aggravation. The overweight might contribute to the deterioration of the imbalance of oxidants/antioxidants both in COPD patients and unaffected individuals.

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