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# TOTAL ANTIOXIDANT CAPACITY OF PLASMA DETERMINED AS FERROUS REDUCING ABILITY OF PLASMA IN PATIENTS WITH COPD.

**S. Emin [1], K. Yordanova[1], D. Dimov[2], V. Ilieva[2], A. Koychev[2], G. Prakova[2], T. Vlaykova[3*]**

[1]Undergraduate students in Medicine from Study group in Biochemistry, Medical Faculty, Trakia University, Stara Zagora
[2]Dept. Internal Medicine, Medical Faculty, Trakia University, Stara Zagora
[3]Dept. Chemistry and Biochemistry, Medical Faculty, Trakia University, Stara Zagora

**ABSTRACT**

**The aim** of the current study was to adapt a method for assessment of total antioxidant capacity of plasma by measuring the ferrous reducing activity of plasma (FRAP method), to determine factors affecting FRAP levels in patients with COPD and in control individuals, and to elucidate the possible effect of FRAP on lung function.

**Methods:** The plasma total antioxidant capacity (TAC) was evaluated in 80 patients with COPD and in 20 non-affected control individuals by FRAP assay.

**Results:** The plasma TAC of patients was significantly lower (0.936 mmol/l $Fe^{2+}$) than that of controls (1.110 mmol/l $Fe^{2+}$). When considered the smoking habits, we found that FRAP levels were significantly lower in patients who were current or ex-smokers compared to those who had never smoked (p=0.030). There was also a tendency for decrease of FRAP levels in patients with advance COPD stage (GOLD IV) than in those with GOLD II or III COPD, as well as in patients with earlier onset of the disease (<65 years). The FRAP levels negatively correlated with the body mass index, as this correlation was more notable in females, both patients and controls.

**Conclusions:** The current results suggest the presence of systemic oxidative stress in COPD and confirm the role of smoking in its aggravation. The overweight might contribute to the deterioration of total antioxidant capacity of plasma especially in women, both with COPD and unaffected once.

**Key words:** COPD, total antioxidant capacity, FRAP, smoking

## INTRODUCTION

The most important non-malignant lung disease, caused by cigarette smokes is chronic obstructive lung disease (COPD), a globally escalating problem in our days (1, 2). Chronic obstructive pulmonary disease is characterized by irreversible airflow limitations and is associated with an abnormal inflammatory response of the lung to noxious particles and gases (3, 4). COPD is a complex disease, which is influenced by genetic factors, environmental influences, common infection of airways and genotype-environmental interactions (5, 6). Cigarette smoking is clearly the major environmental determinant of COPD (7, 8). The increased oxidant burden in smokers derives from the fact that cigarette smoke contains more than $10^{14}$ free radicals/oxidants per puff and is a complex mixture of over 4700 chemical compounds, including aldehydes, quinones, semiquinones, nitrosamines, benzopyrene, and other carcinogens, and it is a risk factor in the development of COPD/emphysema and lung cancer (8-10). Short lived oxidants such as superoxide anion ($O_2^{\cdot -}$) and nitric oxide (NO) are predominantly found in the gas phase. $O_2^{\cdot -}$ and NO react chemically to form highly cytotoxic oxidant peroxynitrite (ONOO–). Free radicals in the tar phase of cigarette smoke are organic in nature, such as long lived semiquinone radicals which can react with O2– to form hydroxyl radical (OH-) and H2O2 (8, 10, 11). Increased oxidative stress in patients with COPD also comes from the increased sequestration of neutrophils in the

***Correspondence to:** *Assoc. Prof. Tatyana Vlaykova, PhD, Medical Faculty, Trakia University, Dept Chemistry and Biochemistry, 11 Armeiska Str., Stara Zagora, 6000, Tel: +35942664326, +359898743832, Fax: +35942700702 e-mail: tvlaykov@mf.uni-sz.bg*

microvasculation which have the potential to release enhanced amounts of ROS (11). Reactive oxygen species (ROS) generated from leucocytes in the blood or air spaces or inhaled in the form of environmental oxidant pollutants are scavenged by antioxidants and antioxidant enzymes. These antioxidant systems include enzymes such as superoxide dismutases, catalase, glutathione peroxidase; proteins such as albumin, ceruloplasmin, and ferritin; and small molecules including ascorbic acid, reduced glutathione, α-tocopherol, ubiquinol-10, cysteine, bilirubin, and uric acid (12, 13).

There is a delicate balance between the toxicity of oxidants and the protective function of the intracellular and extracellular antioxidant defence systems which is critically important for the maintenance of normal pulmonary cellular functions (12). An imbalance between oxidants and antioxidants has been proposed in the pathogenesis of chronic obstructive pulmonary disease (COPD) (10, 14, 15).

The aim of the current study was to adapt a method for evaluation of total antioxidant capacity of plasma by measuring of ferric reducing ability of plasma (FRAP), to explore the FRAP values in patients with COPD and in controls and factors that could affect them and to investigate the possible effect of antioxidant capacity of plasma on lung function.

## MATERIALS AND METHODS

### *Patients' and control populations*

The patient group consisted of 80 patients with COPD aged from 40 to 88 years (median of 68 years. 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 20 healthy voluntaries aged between 23 and 79 years (median of 47 years). The demographic and clinical characteristics of controls and patients, as well as the results of the spirometric examination of patients are presented in **Table 1**.

***Table 1.*** *Demographic and clinical characteristics of controls and patients with COPD enrolled in the current study.*

| Characteristics | Patients with COPD (N) (%) | Controls (N) (%) |
|---|---|---|
| Gender | (80) | (20) |
| males | 60 (75%) | 5 (25%) |
| females | 20 (25%) | 15 (75%) |
| Age at the inclusion in the study | | |
| mean± SD (years) | 66.9±9.3 | 46.8±13.4 |
| median (range) (years) | 68 (40-88) | 47 (23-79) |
| Age at the diagnosis of the disease | | |
| mean± SD (years) | 61.3±9.8 | |
| median (range) (years) | 63 (30-86) | |
| Duration of the disease | | |
| mean± SD (years) | 5.6±5.5 | |
| median (range) (years) | 4 (0-25) | |
| Smoking | (n=77) | (n=18) |
| non-smokers | 22 (29%) | 8 (44%) |
| ex-smokers | 36 (46%) | 3 (17%) |
| current smokers | 19 (25%) | 7 (39%) |
| Smoking habits (packs/year) | | |
| mean± SD (range) | | |
| ex-smokers | 25.9±11.3 (5-60) | 16.7±5.8 (10-20) |
| current smokers | 34.8±15.8 (5-60) | 17.0±4.7 (10-22) |
| all smokers | 29.1±13.6 (5-60) | 16.9±4.7 (10-22) |
| FEV1 % pr. | | |
| mean± SD (range) | 48.20±11.8 (20-79) | |
| FEV1/FVC % | | |
| mean± SD (range) | 58.80±9.4 (37.1-70) | |
| PEF (l/min) | | |
| mean± SD (range) | 261±108 (79-602) | |
| COPD staging (GOLD 2009)(3) | (n=77) | |
| II stage (moderate) | 32 (42%) | |
| III stage (severe) | 41 (53%) | |
| IV stage (very severe) | 4 (5%) | |

***Methods***

**Assessment of plasma TAC**

The total antioxidant capacity of plasma was evaluated by applying the FRAP assay (ferric reducing antioxidant power or ferric reducing ability of plasma) according to the method of Benzie & Strain, 1996 (16). The method is based on the reduction of ferric (Fe3+) to ferrous (Fe2+) ion at low pH. This causes a formation of blue colored ferrous-tripyridyltriazine (Fe2+-TPTZ) complex, which absorbs at 593nm. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma (16, 17). Results were expressed as mmol/l (mM).

The method could be described in brief as the following: the working FRAP reagent was prepared ex tempore by mixing 300 mmol/l acetate buffer, pH 3.6 with 2,4,6-tripyridyl-s-triazine (TPTZ) solution (10 mM in 40 mM HCl) and 20 mmol/l $FeCl_3$ solution in ratio10:1:1 respectively, and was pre-tempered at 37 $^0$ C. The reaction was performed by adding of 100 μl plasma, previously diluted 1:1 with distilled water, to 900 μl FRAP working reagent and the mixture was incubated for 25 min at 37 $^0$ C. The absorbance was measured on 593nm compare to a blank mixture where 100 μl water was added to the working FRAP reagent instead of plasma. Aqueous solutions of known Fe2+ concentration, in range 0.2 to 1 mmol/l ($FeSO_4$. $7H_2O$; Sigma Aldrich, USA) were used for creating of the standard curve. The results were expressed in mmol/l (mM) $Fe^{2+}$.

**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 (FRAP values, age, BMI and spirometric values) was analyzed by the Pearson correlation test. Factors with $p<0.05$ were considered statistically significant.

**RESULTS**

The levels of TAC of plasma determined by FRAP were significantly lower in patients with COPD compared to that of control individuals (0.936±0.344 vs. 1.110±0.235 mmol/l, p=0.035) **(Figure 1)**.

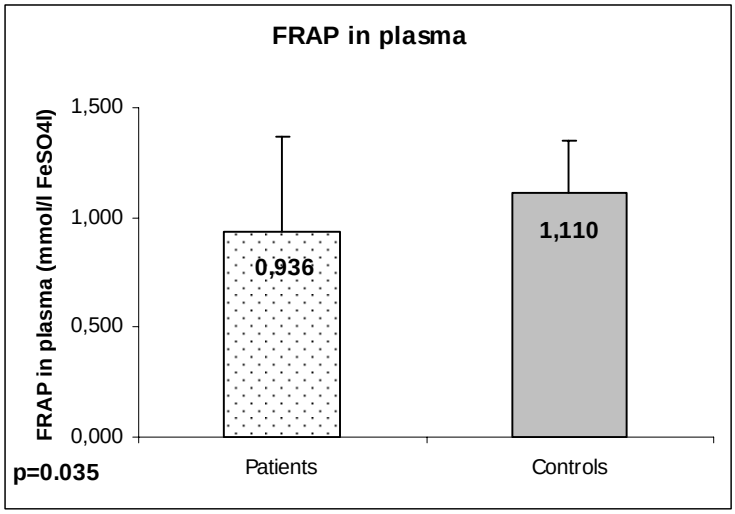


**Figure 1.** FRAP values of patients with COPD and of controls. Data are presented as mean±SD.

On the other hand, FRAP values of patients differed according to the smoking habit: they were significantly lower of smokers (ex- and current smokers together) compared to that of non-smokers (0.891±0.0.324 vs. 1.085±0.389 mmol/l, p=0.030) (Figure 2). However, no difference were found between the FRAP values of smoking and non-smoking controls (p=0.783) (Figure 2). It is interesting to note, that there was no difference in FRAP values between non-smoking controls and patients (p=0.979), whereas a significant difference was seen in FRAP values between controls and patients who smoked (p=0.047) **(Figure 2)**.

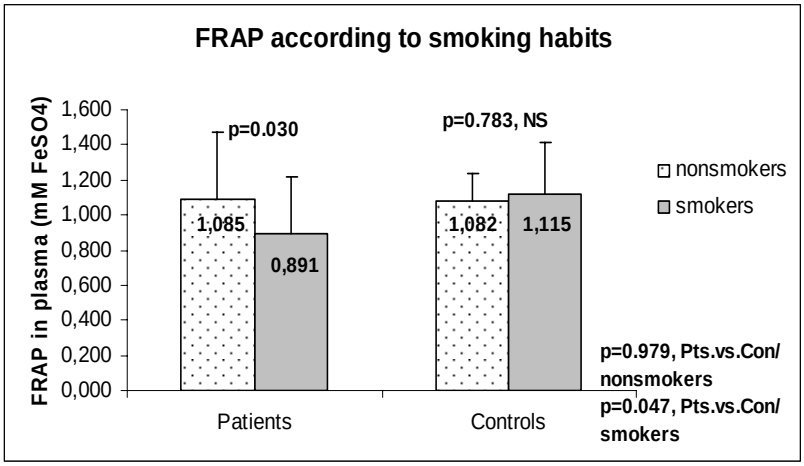


**Figure 2.** FRAP values of patients and controls according to their smoking habits. Data are presented as mean±SD.

Interesting relations were obtained when studying the effect of BMI on the FRAP values. We found a tendency for negative correlation between the BMI and FRAP values in all individuals studied (patients and controls together), but this relation did not reach statistical significance (p=0.122) **(Figure 3A)**. However, when this relation was analyzed in the groups of males and females separately, we found a strong statistically significant negative correlation (R= -0.564) between BMI and FRAP values in females (together patients and controls) (Figure 3B), as well as in the sub-group of female patients (R= -0.542) **(Figure 3D)**. A strong negative correlation, although not significant, was also seen in the sub-group of female controls **(Figure 3C)**. No such correlations were seen in the groups of males, either patients, controls or both together.

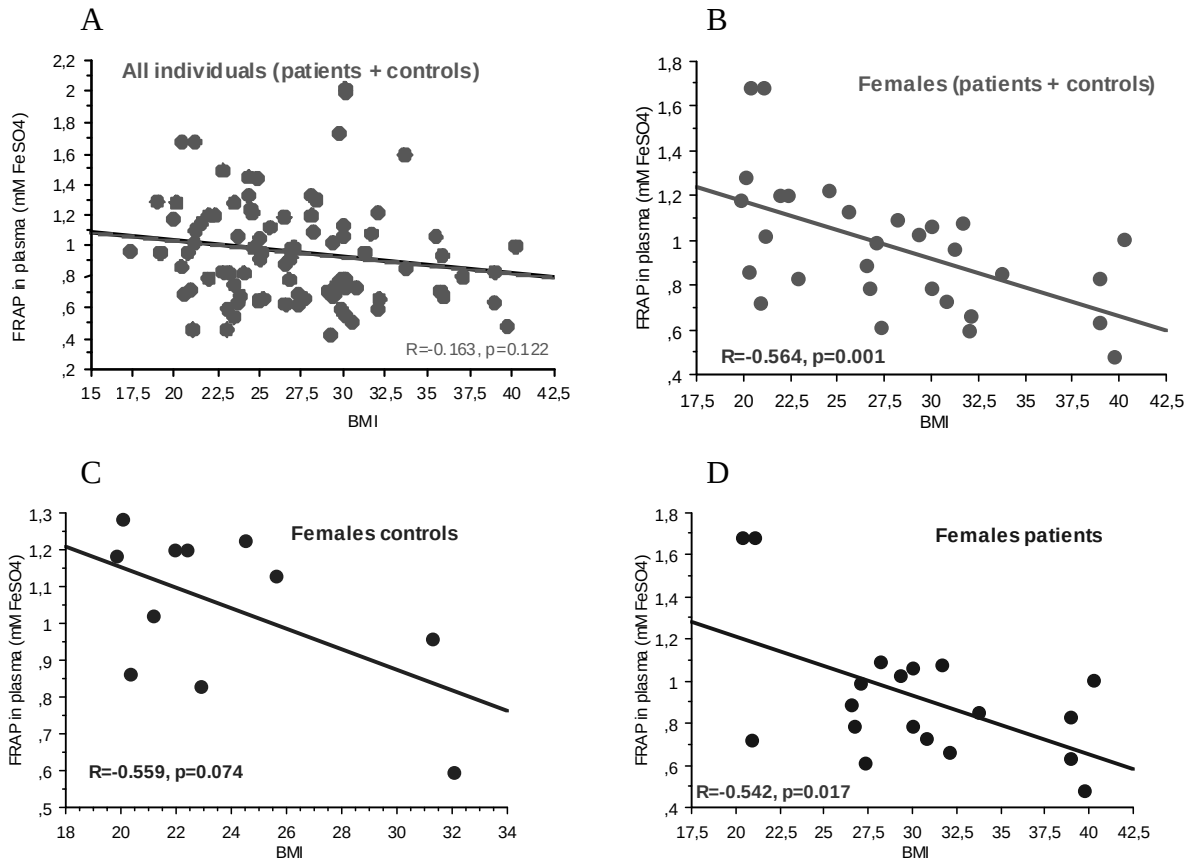


**Figure 3.** Correlations between the BMI and FRAP values of all studied individuals (A), in women (B), in sub-group of female controls (C) and in the sub-group of female patients with COPD (D).

In the current study we aimed to elucidate whether the levels of TAC of plasma (FRAP levels) had some effect on lung function or on other characteristics associated with development and progression of COPD. In the studied population of patients with COPD, there were no statistically significant differences in FRAP levels between patients with different COPD stages, although the levels of this characteristic of plasma antioxidant capacity were apparently lower in patients with very strong severity of the disease in comparison to those with less advanced stages of COPD **(Figure 4)**.

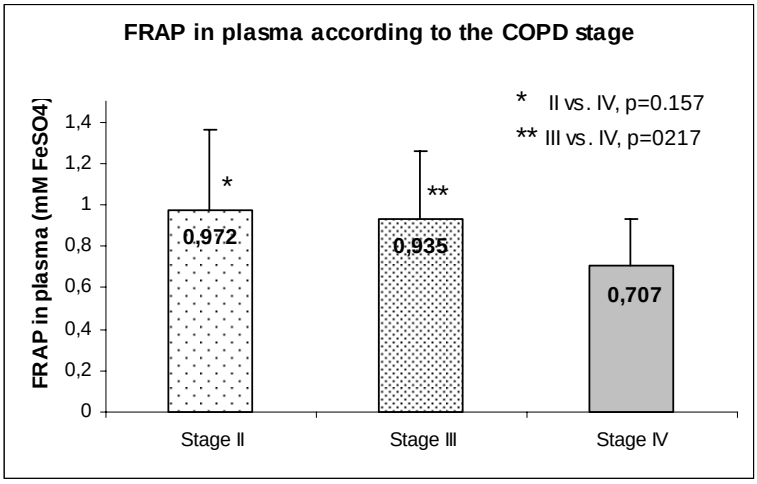


**Figure 4.** FRAP levels of patients with different GOLD stages of COPD. Data are presented as mean±SD.

There were no statistically significant correlations between the FRAP levels and characteristics of lung function obtained during the spirometric examinations of patients with COPD **(Figure 5A and 5B)**.

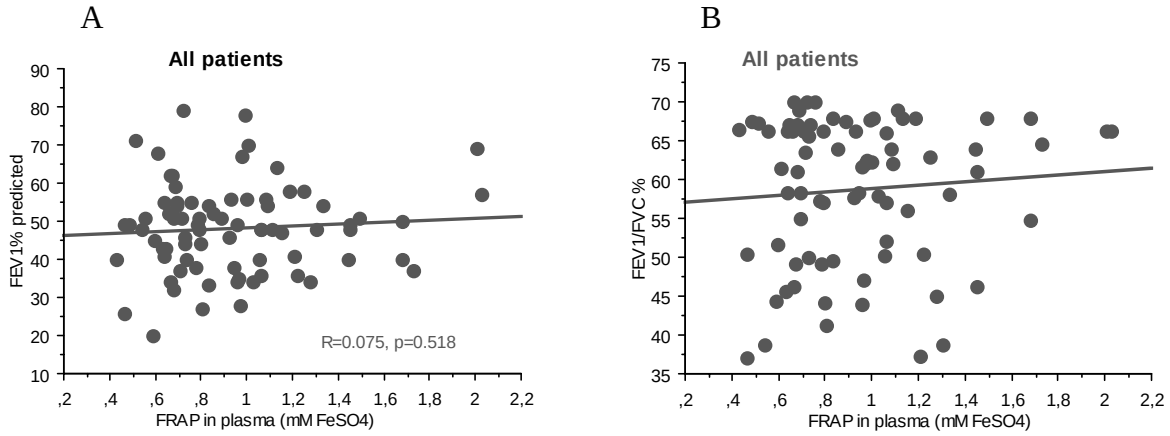


**Figure 5.** Correlations between the FRAP levels and spirometric characteristics (FEV1 % pr. [A] and FEV1/FVC % [B]).

However FRAP levels significantly positively correlated with the age of diagnosis of the disease (R=0.241, p=0.035) (Figure 6A), as this correlation was stronger in patients with severe and very severe COPD (GOLD III and IV) (R=0.398, p=0.007) **(Figure 6B)**.

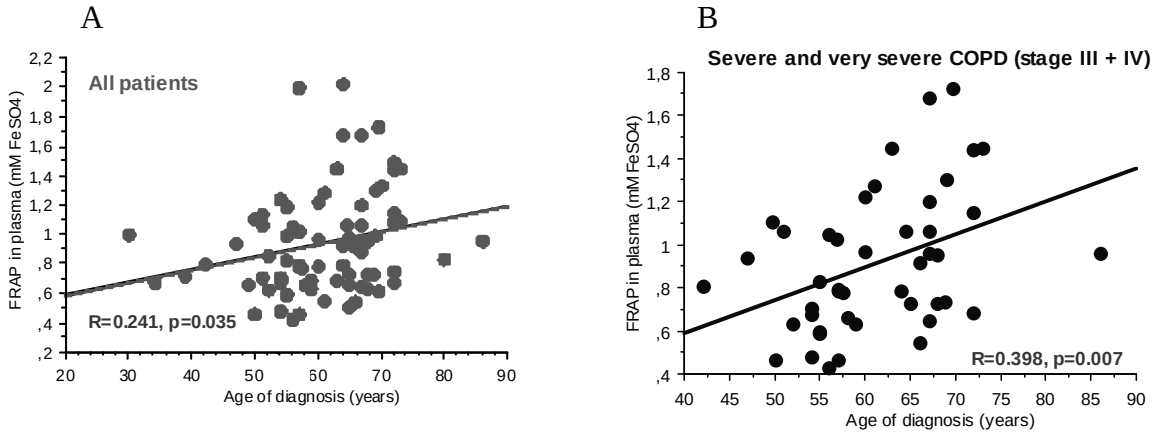


**Figure 6**. Correlations between the FRAP levels and age of the diagnosis of all patients with COPD (A) and of those with severe and very severe COPD (stage III and IV) (B).

In addition we found that the age of the diagnosis of the disease was significantly associated with the smoking habits: smokers developed COPD earlier (mean of 59.3 years) compared to non-smokers (mean of 65.4 years, p=0.011) **(Figure 7)**

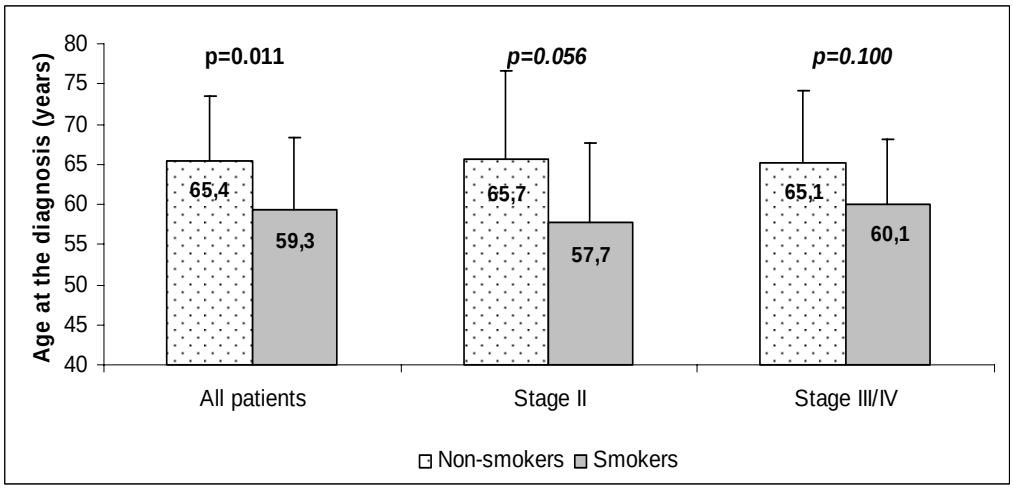


**Figure 7**. Association of the age of diagnosis of COPD and smoking habits. Data are presented as mean±SD

## DISCUSSION

Several assays have been frequently used to estimate antioxidant capacity of biological fluids such as plasma, serum, bronhoalveolar lavage (BAL) fluid etc. or of other biological and food samples. These assays include 2,2-azinobis (3-ethyl-benzothiazolone-6-sulfonic asid) (ABTS, commercialized by Randox Laboratories as Trolox equivalent antioxidant capacity, TEAC assay ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), the oxygen radical absorption capacity (ORAC) and OXY-absorbent test (18-21). Each of these assays has advantages and disadvantages (18, 21), as ORAC has been said to be more relevant because it utilizes a biologically relevant radical source (22) and could determine the antioxidant capacity of both hydrophilic and lipophilic compounds. Another comparative analysis of three different methods has suggested that the antioxidant capacity of human serum followed the order of $ORAC_{total}$ > TEAC (ABTS) > FRAP (23). However the ABTS and ORAC assays have differed among runs (21) suggesting for a lower reproducibility of theses methods. In addition, ORAC technique requires the use of expensive equipment, whereas the other three methods require a simple machine, a spectrophotometer, which is commonly available in most laboratories (21). Another advantages of ABTS

and FRAP is the short time needed for performing of the colored reactions. FRAP values have been shown to be proportional to the reducing power of the mainly nonenzymatic antioxidants in the plasma, mainly uric acid (16, 24) and ascorbic acid (16), but does not detect reduced glutathione (20). Thus, taking into account the high reproducibility, simplicity, rapidity of performance and the high correlation with ascorbic acid, uric acid and other antioxidants, FRAP technique have been suggested as the most appropriate technique for determining antioxidant (20, 21).

In this respect, in the current study we chose FRAP assay to determine the total antioxidant capacity (TAC) of plasma of patients with COPD. The FRAP values of plasma of controls varied between 0.597 and 1.600 mmol/l with a median of 1.179 mmol/l) while those values were 0.428 and 2.025 mmol/l and median of 0.847 mmol/l for patients with COPD. The obtained values of FRAP assay are commensurable to those reported for 44 workers from Iran with a rotational shift schedule (25), for 70 healthy subjects from the Netherlands aged 18-58 years (26), and for apparently healthy Chinese adults studied by Benzie et al. (16). In contras, values around 0,50- 0,57 mmol/l were found in plasma samples from unselected Italian outpatients (20) and another group f healthy Italian subjects (Gerardi G, 2002).

When FRAP values were compared between controls and COPD patients, we found a significant reduction of plasma TAC of patients with COPD than of control individuals. Our finding is in line to that obtained by Nadeem et al. for Indian patients with COPD (4). Similarly, significant reduction of the total antioxidant capacity of plasma, however assessed by Troxol equivalent antioxidant capacity (TEAC) was reported for COPD patients from UK (23) and for patients with COPD from Turkey using a novel automated method (27). Our results also showed that smoking significantly affected the TAC of plasma of patients leading to markedly reduction of the values. There are several reports describing analogous effect of smoking on TAC either in control individuals or in patients (23, 27, 28). We also found that smoking was significantly associated with younger age of the diagnosis of COPD. Based on this observation and having in mind the next finding of our study describing an inverse relationship between the age of diagnosis and FRAP values, we may conclude that development of COPD occurs in early age of smokers with lower total antioxidant capacity.

In our study, we also obtained an interesting inverse correlation between the BMI and the FRAP values, especially in the women, both controls and patients with COPD. There are several other investigations that have explored the association between the body weight or obesity and total antioxidant capacity in different individuals' group and conditions. Thus, in a large study that has comprised apparently healthy 1514 men and 1528 women from the Attica area in Greece, there was found an inverse relationship between body fat, central adiposity and antioxidant capacity, irrespective of age and various other potential confounders, namely smoking, physical activity, dietary habits, blood pressure, glucose levels, and lipid concentrations (29). Similarly, in a study with 68 children without co-morbidities the obesity was associated with oxidative stress (30). In addition, an increase of the plasma TAC was found to be related to a reduction in body weight after 3 years of intervention in a high cardiovascular risk population with a Mediterranean-style diet rich in virgin olive oil (31). Moreover, the plasma TAC values were found to be impaired in morbidly obese patients and the weight loss from an intragastric balloon was associated with significant increase in plasma TAC values (32). Thus, our results confirmed the suggestion done by other investigators that overweight and obesity may induce oxidative stress and decreased on antioxidant capacity of plasma.

In conclusions based on the results of our current study we suggest the presence of systemic oxidative stress in COPD and confirm the role of smoking in its aggravation. The overweight might contribute to the deterioration of total antioxidant capacity of plasma especially in women, both with COPD and unaffected once.

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