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Serum levels of oxidative stress as a marker of disease severity in idiopathic pulmonary fibrosis

Zoe D. Daniil*, Evangelia Papageorgiou, Agela Koutsokera, Konstantinos Kostikas, Theodoros Kiropoulos, Andriana I. Papaioannou, Konstantinos I. Gourgoulianis

Department of Respiratory Medicine, University Hospital of Larissa, University of Thessaly, Larissa, Greece

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a fatal illness characterized by progressive fibrosis resulting in severe dyspnea and impairment of lung function. Although the mechanisms by which lung fibrosis develops are not fully ascertained, recent findings suggest that oxidative stress may play an important role in the pathogenesis of tissue fibrosis.

Aim: To evaluate the oxidative stress in the serum of patients with IPF and to explore the relationship between oxidative stress levels, dyspnea and impairment of lung function.

Material and methods: Blood samples from 21 untreated patients with IPF, sequentially recruited over a period of 2 years, and 12 controls were analyzed. The level of oxidative stress in the blood was determined through a spectrophotometric procedure (D-ROMs test). FVC and DLCO were measured in all patients. The level of dyspnea was assessed by the Medical Research Council (MRC) chronic dyspnea scale.

Results: Serum levels of oxidative stress were significantly increased in patients with IPF compared to controls (mean $\pm$ SEM: 356.8 ± 14 and 201 ± 10 Carratelli units respectively, $p < 0.001$). Oxidative stress was negatively associated with FVC ($p < 0.01$, $r = -0.79$) and with DLCO ($p < 0.01$, $r = -0.75$). Furthermore, oxidative stress was significantly correlated with MRC dyspnea score ($p < 0.01$, $r = 0.87$). Oxidative stress measurements were highly reproducible on two consecutive measurements in the same patients.

Conclusion: The levels of systemic oxidative stress are enhanced in patients with IPF and could provide useful information about the classification of IPF severity. Strategies to reduce the oxidant burden in IPF may be beneficial in reducing the progressive deterioration of these patients.

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Keywords: Idiopathic pulmonary fibrosis; Oxidative stress; Medical research council chronic dyspnea scale; Lung function

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating condition that leads to progressive lung destruction and scarring. The diagnosis of IPF requires compatible clinical history, functional and high-resolution computed tomography (HRCT) findings, and the exclusion of other known causes of interstitial lung disease [1]. Usual interstitial pneumonia (UIP) is the histopathologic pattern that identifies patients with IPF in lung biopsy specimens [1]. UIP has patchy lung involvement with a variable stage of fibrosis (active fibrogenesis occurring in the so-called fibroblastic foci) and a low grade of inflammation [2].

It has been widely held that pulmonary fibrosis begins with alveolar inflammation and that chronic inflammation modulates fibrogenesis [3]. However, recent observations have led to new concepts in the pathogenesis of IPF. Several key observations suggest that inflammation does not play a prominent pathogenetic role, and that alveolar epithelial injury directly results in lung fibrosis [4]. Furthermore, ultrastructural studies have demonstrated alveolar type II cell injury and apoptosis in lung biopsies from patients with IPF [5]. A mechanism proposed to explain epithelial cell apoptosis is an increased production of oxidants in IPF. Additionally, free radicals activate

*Corresponding author. Tel.: + +30 2410682898.
E-mail address: zdaniil@med.uth.gr (Z.D. Daniil).

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transforming growth factor-β (TGF-β), one of the most important growth factors in the pathogenesis of fibrotic lung diseases, which promotes epithelial cell apoptosis [6,7].

There are numerous studies showing that the oxidant burden in the lungs of the patients with pulmonary fibrosis is increased and that inflammatory cells of these patients generate more radicals than the cells of healthy control subjects [8–10]. Moreover, indicators of free radical activity are increased in the serum of patients with IPF [11,12]. The aim of the current study was to evaluate the systemic levels of oxidative stress in the serum of patients with IPF using a commercially available and easy to perform assay and to explore the relationship between the levels of oxidative stress, and the level of dyspnea and impairment of lung function in such patients.

2. Methods

2.1. Patients with IPF

The population studied consisted of 21 patients (15 males, aged 53–80 yr) with clinical and radiological features of IPF. They were recruited sequentially from the respiratory outpatient clinic over a period of 2 years. All patients fulfilled the criteria for the diagnosis of IPF established by the American Thoracic Society, the European Respiratory Society, and the American College of Chest Physicians [1]. Histological features of UIP were confirmed by video-assisted thoracoscopic biopsy in 8 patients. Five patients were never smokers and 16 were ex-smokers. The time elapsed from the time of diagnosis to the present study was 13 ± 1 months. All patients had been off treatment for at least 2 months when oxidative stress measurement was performed. None of the patients presented significant comorbidities that might influence the systemic levels of oxidative stress. The study protocol was approved by the local ethics committee and informed consent was obtained from each patient.

2.2. Normal subjects

Twelve normal individuals (matched for age, sex and smoking status) were evaluated (8 males, aged 43–82). All had normal chest X-rays and normal lung function tests.

2.3. Pulmonary function tests

Lung function tests, including FEV_1, FVC, FEV_1/FVC ratio, total lung capacity (TLC), residual volume (RV) and DLCO, were performed within 1 week of the measurement of serum oxidative stress. TLC and RV were measured by the helium dilution method with a Master Screen apparatus (Erich Jaeger GmbH, Wuerzburg, Germany), and DLCO by the single breathholding helium dilution method. Measurements were expressed as percentages of predicted values [13,14]. In all patients, the arterial PaO_2 and $PaCO_2$ were also measured at rest and on the same day with oxidative stress measurement.

2.4. Dyspnea

Dyspnea was assessed by the modified MRC chronic dyspnea self-administered questionnaire that consists of six questions about perceived breathlessness: category 0, no dyspnea; category 1, slight degree of dyspnea (troubled by shortness of breath when hurrying on the level or walking up a slight hill); category 2, moderate degree of dyspnea (walks slower than people of the same age on the level because of breathlessness); category 3, moderately severe degree of dyspnea (has to stop because of breathlessness when walking at own pace on the level); category 4, severe degree of dyspnea (stops for breath after walking about 100 yards or after a few minutes on the level); category 5, very severe degree of dyspnea (too breathless to leave the house or breathless when dressing or undressing) [15].

2.5. HRCT

The HRCT examination was performed within a 1-month interval from the pulmonary function tests and lung biopsy. The CT scans were performed using either a Somaton HiQ or a Somaton Plus scanner (Siemens, Erlanger, Germany). Scans were performed with 1–1.5 mm section thickness and a 1–2 s scanning time during breath holding at end inspiration. An experienced radiologist, without any knowledge of the clinical and functional findings, examined the HRCT scans and an assessment made of presence of a pattern consistent with IPF.

2.6. Assessment of levels of oxidative stress

Blood samples were collected from all IPF patients and control subjects and were analyzed immediately after their collection. A commercially available method was used for the assessment of the levels of oxidative stress in the blood (d-ROMs test; Diacron, Grosseto, Italy), as has been previously described [16,17]. This is a spectrophotometric method that assesses overall oxidative stress by measuring total hydroperoxides, given that hydroperoxides are intermediate oxidative products of lipids, peptides, and amino acids. Immediately after the collection the blood is centrifuged at $1500 \times g$ for 15 min at 4 °C and 20 μL of the serum is diluted in 1 mL acetate buffered solution (pH = 4.8, R1 Reagent). Hydroperoxide groups react with the transition metal ions liberated from the proteins in the acidic medium and are converted to alkoxyl and peroxyl radicals according to Fenton's reaction [18]. These newly formed radicals, the quantities of which are directly proportional to peroxides present in the serum, are trapped chemically with 20 μL of chromogen (*N*,*N*-diethparaphenyldiamine, R2 Reagent) leading to the formation of the radical cation of this chromogen. The purple color

resulting from this reaction over time was monitored in a spectrophotometer (Perkin-Elmer λ16 Norwalk, Connecticut, USA) at 505 nm. The results of this method are expressed in conventional units (Carratelli Units, UCarr); 1 UCarr corresponds to 0.8 mg/L H_2O_2.

2.7. Reproducibility

The repeatability of the measurements of hydroperoxides with the d-ROMs test in the serum was checked on serum samples obtained from two serial blood collections performed on two consecutive days in six patients with IPF and four controls. A further analysis consisted of two serial measurements on the initial (1st day) samples, in order to assess the reproducibility of the method on the same samples obtained from the 10 previously mentioned patients.

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Comparisons between the study groups were performed using the Mann–Whitney *U* test. Correlations between different parameters were determined by Spearman's rank correlation coefficient. Normality of distribution of the data was checked with Shapiro–Wilk's test. For the evaluation of the reproducibility of the measurements of oxidative stress in serum we have used the method described by Bland and Altman [19]. *P*-values of less than 0.05 were regarded as significant. Analysis was performed using the SPSS 11.5 statistical package.

3. Results

All IPF patients complained for some degree of dyspnea (MRC score > 0) and all were ambulatory (nobody with MRC score of 5). There were 5 patients with MRC grade 1 dyspnea, 5 patients with MRC grade 2 dyspnea, 7 patients with MRC grade 3 dyspnea and 4 patients with MRC grade 4 dyspnea. Lung function data are shown in Table 1. All patients had a restrictive lung function pattern characterized by a FEV_1/FVC ratio $\geqslant$ 75%. The DLCO and FVC were also decreased in all patients. Finally, the HRCT of the chest revealed the typical findings of IPF in all patients [1]. No emphysema was evident in the HRCTs. The study populations did not differ significantly in terms of sex, age, or smoking habits (Table 1). Lung function parameters were significantly reduced in the IPF group ($p < 0.001$ in all comparisons).

The serum levels of oxidative stress in the control group was 201 ± 10 UCarr. In the IPF group a highly significant ($p < 0.001$) increase in serum level of oxidative stress was noted (356 ± 14 UCarr; Fig. 1). No significant differences were observed between ex-smokers and never smokers in the levels of oxidative stress.

Table 1
Characteristics of the study populations

Parameter	Control	IPF*
N	12	21
Sex, male/female	8/4	15/6
Age, yr	70 ± 1.9	65 ± 3.8
Ex-smoker/nonsmoker	9/3	16/5
FVC (% predicted)	96.2 ± 3.9	64.4 ± 3.3†
DLCO (% predicted)	92.6 ± 1.9	51 ± 3.8†
PaO_2 (mmHg)	85.5 ± 2.5	73 ± 4†
$PaCO_2$ (mmHg)	39 ± 1.4	36 ± 3

Definition of abbreviation: IPF = idiopathic pulmonary fibrosis.
*Significant differences between the groups: †$p < 0.01$.

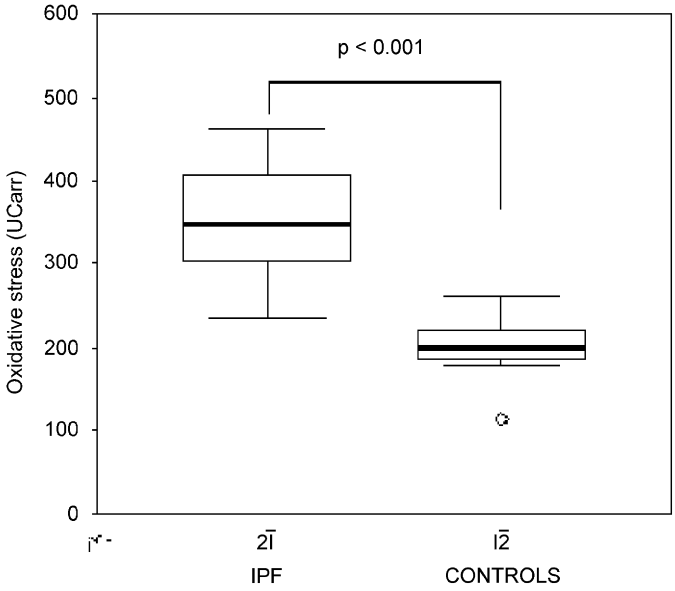

Fig. 1. Oxidative stress level in controls and IPF patients. The mean is indicated by the horizontal bars. The two groups differ significantly as indicated.

3.1. Correlations

In the IPF group a highly significant, positive correlation between the serum level of oxidative stress and the severity of dyspnea, as reflected by the MRC chronic dyspnea score (Fig. 2, $p < 0.01$, $r_s = 0.87$) was observed. Additionally, a negative correlation was obtained between the serum levels of oxidative stress and the impairment of lung function, as reflected by the FVC in the same patients (Fig. 3, $p < 0.01$, $r_s = -0.79$) and the DLCO (Fig. 4, $p < 0.01$, $r_s = -0.75$). No significant correlations were observed between oxidative stress levels and the FEV_1, the FEV_1/FVC ratio, the PaO_2 or the $PaCO_2$. Additionally, no significant correlations were observed between the levels of oxidative stress and lung function parameters in the control group.

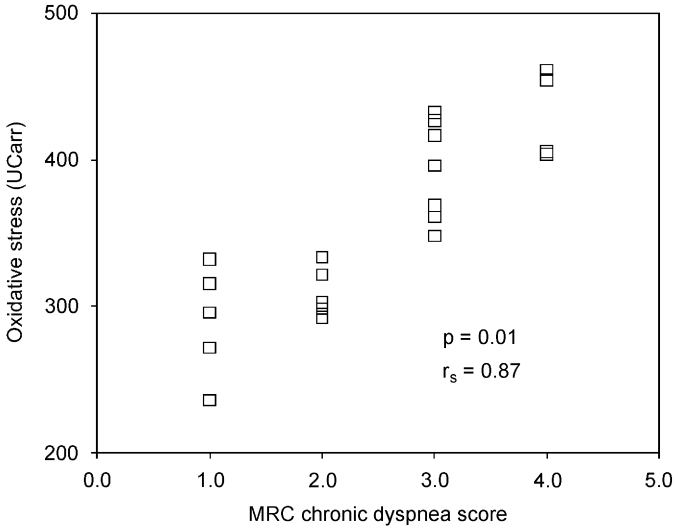

Fig. 2. Relationship between serum oxidative stress level and MRC chronic dyspnea score in patients with IPF. There is a significant, positive correlation between oxidative stress level and MRC chronic dyspnea score ($p<0.01$, $r_s = 0.87$).

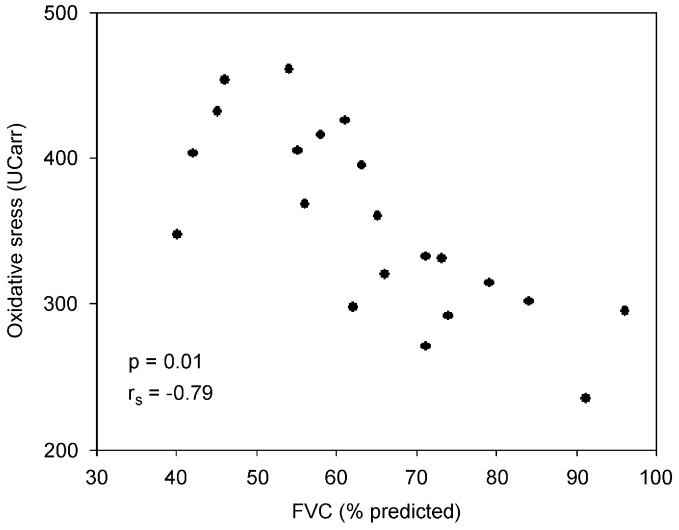

Fig. 3. Relationship between serum oxidative stress levels and FVC in patients with IPF. There is a significant, negative correlation between oxidative stress level and FVC ($p<0.01$, $r_s = -0.79$).

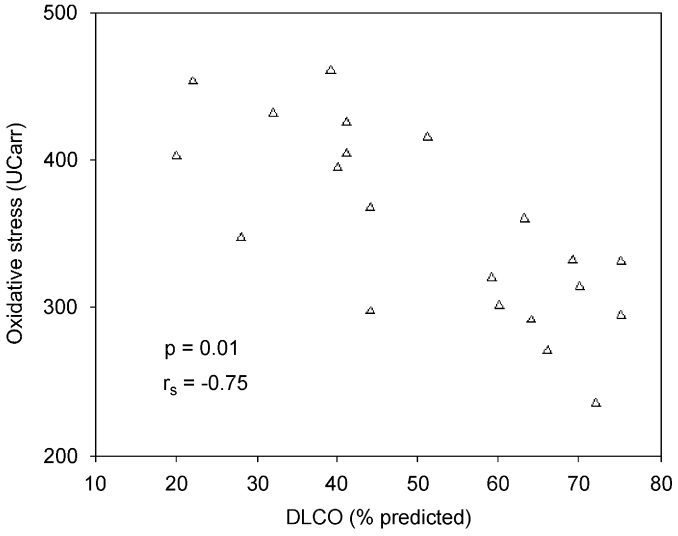

Fig. 4. Relationship between serum oxidative stress level and DLCO in patients with IPF. There is a significant, negative correlation between oxidative stress level and DLCO ($p<0.01$, $r_s = -0.75$).

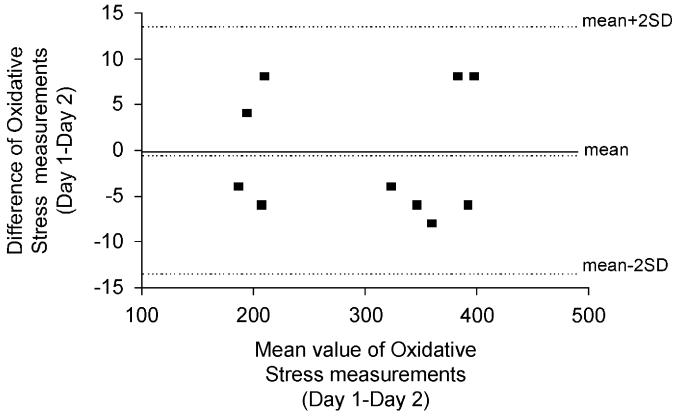

Fig. 5. Assessment of repeatability of oxidative stress measurements in two consecutive days in 10 subjects, presented in a Bland and Altman plot (differences against mean values). The levels of oxidative stress in the plots are expressed in Carratelli units (UCarr). Dotted lines represent the mean difference value and the limits of agreement (± 2 SD).

3.2. Reproducibility and repeatability of oxidative stress measurements

The measurements of oxidative stress in two different blood samples taken on two consecutive days from patients with IPF and controls presented excellent repeatability. Oxidative stress levels on days 1 and 2 were 300 ± 89 and 301 ± 90 UCarr, respectively. The correlation between oxidative stress measurements on two consecutive days was statistically significant ($r = 0.99$, $p < 0.0001$). The mean difference with limits of agreement was 0.60 ± 13.46 (mean $\pm$ 2SD) and all values were well within the limits of agreement in the Bland and Altman plot (Fig. 5). Similarly, two consecutive measurements of the same samples taken from the same subjects on the first day presented excellent repeatability (data not presented).

4. Discussion

In the present study we have demonstrated that systemic oxidative stress is significantly elevated in patients with IPF compared with healthy controls. Furthermore, the levels of oxidative stress in serum present significant correlations with the levels of dyspnea as expressed with MRC chronic dyspnea scale, as well as with the functional parameters expressing the severity of the disease, i.e. the FVC and the DLCO. The fact that serum oxidative stress was significantly correlated with the impairment of important lung

function parameters such as FVC and DLCO and with the level of dyspnea, suggests that it may be useful in serological assessment of disease severity in IPF and might reflect different stages of disease.

Previous studies have shown that various markers of oxidative stress are increased in the lung, including hydrogen peroxide and 8-isoprostane. Such markers have been determined in various biological samples, as in blood [20,21], sputum [22], BAL [23] and exhaled breath condensate [24,25] collected from patients with lung diseases. These samples express either local or systemic levels of oxidative stress. Reactive oxygen species (ROS) are produced by multiple mechanisms in all cell compartments and play a significant role in the pathogenesis of lung diseases where oxidative stress is increased [26]. Rahman and colleagues have found that a profound systemic oxidant/antioxidant imbalance occurs in patients with IPF, which also reflects local oxidative stress measured in bronchoalveolar lavage fluid (BALF) [12]. Moreover, 8-isoprostane (8-epi-prostaglandin F2 alpha) (a biomarker of lipid peroxidation due to reactive oxygen species) is enhanced in BALF from patients with interstitial lung diseases. However, the same investigators did not observe any correlation between the levels of 8-isoprostane and lung function parameters [27]. Interestingly, Jack and colleagues have found that lipid peroxidation is increased in the serum of patients with IPF and is associated with clinically deteriorating disease. However, their findings should be interpreted with caution because of the methodological limitations of their study. There is concern about the selection criteria of control subjects and their influence on the study findings. Further evidence of increased oxidative stress comes from the Saleh and colleagues' study showing increased production of the nitric oxide (NO) radical due to the induction of inducible NO synthase (INOS) in the lungs of patients with IPF [28].

In the present study we have indirectly assessed the systemic oxidative stress in the serum of patients with IPF by measuring total hydroperoxides in blood by a commercially available method called d-ROMs test. This method has been validated before in blood samples [17]. In blood samples it has been shown that the method presents both an acceptable stability and an acceptable margin of error [29]. Furthermore, in our study the measurement of overall oxidative stress was highly repeatable on the same samples and on samples taken from the same subjects on two consecutive days, thus indicating that the results may be safely interpreted in each patient.

The source of oxidative stress in the serum of patients with IPF is not currently known. Rahman and colleagues have speculated that it may be derived from the release of ROS from activated alveolar and peripheral neutrophils and macrophages [12]. Additionally, elevated serum levels of superoxide dismutase have been found in patients with IPF and it has been proposed that these increased levels suggest an increased oxidant burden released from activated neutrophils, as a part of the pathogenesis of the disease [30]. There are numerous studies showing that the oxidant burden in the lungs of the patients with pulmonary fibrosis is increased and that inflammatory cells of these patients generate more radicals than the cells of healthy control subjects [12,27]. Moreover, indicators of free radical activity are increased in the serum of patients with IPF [11,12]. In contrast to the study by Montuschi et al. who did not find any correlation between 8-isoprostane levels and functional parameters [27], we have presently found significant correlations between the levels of systemic oxidative stress and functional parameters expressing disease severity in IPF, as well as with the levels of dyspnea. These findings may have implications about the overall systemic oxidative stress in IPF, as they suggest that a simple serum assay may represent a good indicator of the functional status of such patients. Interestingly, our study population consists of patients with no significant comorbidities that might influence the systemic levels of oxidative stress. An emerging body of literature that has accumulated in recent years suggests that alveolar type II cell injury and apoptosis may be an important early feature in the pathogenesis of pulmonary fibrosis [31]. Ultrastructural studies have demonstrated alveolar type II cell injury and apoptosis in lung biopsies from patients with IPF [5]. Among the mechanisms proposed to explain the epithelial cell apoptosis is increased production of oxidants in IPF. Several studies have shown excessive oxidant production in IPF and deficiencies in glutathione production [28,32,33]. Finally, a recent multicenter study has shown that acetylcysteine, a precursor of the major antioxidant, glutathione, given at a daily dose of 1800 mg, restores depleted pulmonary glutathione levels and resulted in a statistically significant improvement in lung function in IPF patients [34]. This study provides further rationale for the assessment of oxidative stress status in patients with IPF.

In summary, our results imply that an increased systemic oxidative stress occurs in patients with IPF, an observation that could suggest a possible role for oxidative stress in the pathogenesis of pulmonary fibrosis. Since the level of systemic oxidative stress, assessed with a rapid and high repeatable method, correlated significantly with both functional (FVC, DLCO) and clinical indices (the MRC chronic dyspnea score) of disease severity, it could be a useful adjunct in the assessment of the clinical status of patients with IPF. However, because of the relatively small size of the population studied, further studies are needed to support our findings. In addition, it would be useful to validate the predictive value of systemic oxidative stress in the disease progression and survival.

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