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# Oxidative stress and inflammation parameters-novel biomarkers for idiopathic pulmonary fibrosis

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**Abstract. – OBJECTIVE: The pathophysiological mechanisms of idiopathic pulmonary fibrosis (IPF) are not well elucidated. It is assumed that oxidative stress and inflammation are the key underlying culprits for its onset and progression. To gain deeper insight into these processes, we have evaluated several oxidative stress parameters, inflammation markers [i.e., high sensitivity C-reactive protein (hsCRP), serum amyloid A1 (SAA1)], soluble programmed cell death-ligand 1 (sPD-L1), and 25-hydroxyvitamin D [25(OH)D] in IPF patients.**

**PATIENTS AND METHODS: Biochemistry analyses were done in 30 consecutive IPF patients and 30 age and gender-matched healthy control group (CG).**

**RESULTS: IPF patients had significantly higher advanced oxidation protein products ($p<0.001$), pro-oxidant-antioxidant balance ($p=0.010$), total oxidative status ($p<0.001$), and ischemia modified albumin ($p<0.001$) compared to CG. Lower total antioxidant status and total sulfhydryl groups (tSGH) and significantly higher sPD-L1, hsCRP ($p<0.001$ for all), SAA1 proteins ($p=0.014$) and [25(OH)D] severe deficiency [11.0 (9.6-15.1) nmol/L] in IPF patients compared to CG were observed. Paraoxonase 1 activity and hsCRP level were lower, while tSHG and sPD-L1 were higher in IPF patients with more severe disease (i.e., II+III stage compared to I stage, $p<0.05$ for all).**

**CONCLUSIONS: IPF patients are in a state of profound oxidative stress compared to healthy people. The inflammatory component of the disease was confirmed by higher hsCRP and SAA1, but lower [25(OH)D] in IPF than in healthy people. Also, higher levels of sPD-L1 in patients with IPF compared to healthy individuals suggest that sPD-L1 may have a significant role in immune response in IPF.**

*Key Words:*
Idiopathic pulmonary fibrosis, Oxidative stress, Inflammation, Soluble programmed cell death-ligand 1.

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a rare, chronic, and fatal disease of unknown cause, with an estimated incidence of 2.8-19/100 000 people per year, in Europe and North America[1,2]. There is an association between IPF and some well-known factors, such as gender (male), smoking cessation, environmental risks factors, microbes, and comorbidities. Clinical characteristics of this disease are fibrosing interstitial pneumonia, dyspnoea, and worsening of lung function[3]. Repetitive injury to the alveolar epithelium and activation of aberrant immune, and inflammatory signalling results in lung remodelling, causing pulmonary fibrosis[4,5].

Oxidative stress (OS) covers molecular, cellular, and tissue abnormalities as the consequences of the imbalance between reactive oxygen species (ROS) production and antioxidant defence in favour of ROS. The lung is particularly vulnerable to OS because of exposition to the highest levels of oxygen[6,7]. Exogenous oxidants increase oxidant production and activate inflammatory cells to generate free radicals, favouring fibrotic interstitial lung reactions[7]. Redox-altering therapeutic strategies are discussed for the finding future biomarkers and therapeutic development in IPF.

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IPF has been considered an inflammatory disease for a long time. This concept was changed following the negative results of multiple studies where anti-inflammatory therapy was not successful[8]. Today, there is evidence[9] of common biology for IPF and lung cancer. However, despite all the research and findings, there are still extremely limited data according to the pathogenesis of this disease.

Since pathophysiological mechanisms of IPF are not well elucidated, to obtain deeper knowledge into these processes we aimed to estimate several OS status parameters, inflammation markers [i.e., high sensitivity C-reactive protein (hsCRP), serum amyloid A1 (SAA1)], hydroxyvitamin D [25(OH)D] status, as well as soluble programmed cell death-ligand 1 (sPD-L1)] in IPF patients. Importantly, studies that investigated OS status used different markers for its estimation. Additionally, to the best of our knowledge studies that investigated SAA1 and sPD-L1 regarding the mentioned pathology are scarce.

## Patients and Methods

The study was approved by the School of Medicine Belgrade University Ethics and Review Board (No. 61206-2299/2-17) and all patients provided written informed consent. Plasma samples were obtained from 30 eligible consecutive patients at the initial medical check-up when the main diagnosis of IPF was confirmed, hospitalized in Clinic for Pulmonology, Clinical Centre of Serbia, in a period between June 2017 to June 2018 and 30 healthy subjects of similar age and gender distribution (control group, CG). Accordingly, participants that met the criteria for IPF diagnosis were included in the study. Exclusion criteria were: malignancies, endocrine disorders, liver and kidney diseases, acute inflammation, autoimmune diseases. Patients were categorized by the number of comorbidities (0, 1-2, and ≥3), so as according to GAP stages (I, II, III stage)[10].

### Biochemistry Analyses

Blood samples were collected into lithium-heparin vacutainer tubes (BD Diagnostics, Wokingham, UK). Plasma was separated by centrifugation at 1000xg RCF, for 15 minutes and stored at -80°C, until analysis.

We measured glucose, total proteins, albumin, total cholesterol, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) and triglycerides, OS status parameters [advanced oxidation protein products (AOPP), pro-oxidant-antioxidant balance (PAB), total oxidative status (TOS), total antioxidant status (TAS), total sulfhydryl groups (tSHG), paraoxonase 1 (PON1) activity, ischemia modified albumin (IMA), malondialdehyde (MDA)], inflammation markers [hsCRP and SAA1], sPD-L1 and [25(OH) D] in the blood of study subjects. Due to limited financial resources we were not able to measure vitamin D concentration in healthy controls.

According to laboratory [25(OH)D] reference values were as follows: severe deficiency <25 nmol/L, deficiency 25-50 nmol/L, moderate deficiency 50-75 nmol/L, optimal > 75 nmol/L.

Parameters were selected to reflect pro-oxidative processes in patients' circulation, as well as their antioxidative capacity, and were measured as we previously reported[11-13]. In brief, MDA levels were determined as a thiobarbituric acid reactive substance[14]. AOPP levels were determined by the method of Witko-Sarsat using a reaction with potassium iodide and glacial acetic acid[15]. PAB levels were measured using 3,3',5,5'-tetramethylbenzidine as a chromo gen by the method of Alamdari et al[16]. IMA was determined by the method of Bar-Or et al[17] and values are reported as absorption units (ABSU). TOS was measured with ABTS as chromogen[18]. TAS was measured with o-dianisidine[19]. tSHG levels were measured using dinitrodithiobenzoic acid as a reagent in the alkaline buffer by the method of Ellman[20]. PON1 activity was determined by the method of Richter and Furlong[21] with a substrate-paraoxon.

For the sPD-L1 and SAA1 determination in human plasma, DuoSet ELISA system (R&D systems Europe, Ltd. Abingdon, UK) was used, as a sandwich enzyme-linked immunosorbent assay (ELISA).

### Statistical Analysis

Analyzed biomarkers' distributions deviated from normal, Gaussian distribution, and its average values were presented as medians and $25^{th}$-$75^{th}$ percentile values. Accordingly, we implemented non-parametric methods – Mann-Whitney U test for two groups and Kruskal-Wallis ANOVA for the three-group comparison. Multiple linear regression (MLR) analysis was applied to find the parameters with the highest influence on variability in sPD-L1. All parameters that entered MLR analysis were logarithmically transformed. Analyses were carried out using SPSS 18.0 (IBM, Armonk, NY, USA). All tests were 2-sided, and a $p$-value <0.05 was considered statistically significant.

## Results

Basic clinical and biochemical data in the group of IPF patients and CG are presented in Table I.

Also, OS status parameters in healthy people and IPF patients are presented in Table I. Patients with IPF exhibited higher OS compared to CG, evidenced by the significantly higher levels of AOPP ($p$<0.001), PAB ($p$=0.010), and TOS ($p$<0.001). IMA was also significantly higher in patients than in CG ($p$<0.001). The level of MDA was similar in both groups ($p$=0.369).

At the same time, IPF patients had a lower level of antioxidative potency, reflected by the significantly lower TAS ($p$<0.001) and tSHG ($p$<0.001). PON1 activity was similar in both groups ($p$=0.316).

**Table I.** General, clinical and biochemical characteristics of the study subjects.

| Parameter | CG | IPF | *p*-value |
|---|---|---|---|
| Age (years) | 62.5 ± 5.6 | 66.4 ± 9.2 | 0.123 |
| Gender, n | | | |
| male | 9 | 10 | 0.852* |
| female | 21 | 20 | |
| BMI (kg/m$^2$) | 27.2 ± 4.4 | 26.7 ± 5.3 | 0.840 |
| Smoking status | | | |
| Ex-smokers | 2 | 12 | |
| Active smokers | 7 | 6 | |
| Non-smokers | 21 | 12 | 0.008* |
| Disease stadium, n (%) | / | | |
| 1$^{st}$ | | 13 (43.3) | |
| 2$^{nd}$ | | 13 (43.3) | |
| 3$^{rd}$ | | 4 (13.3) | / |
| Comorbidities, n (%)** | / | | |
| 0 | | 3 (10) | |
| 1-2 | | 15 (50) | |
| ≥ 3 | | 12 (40) | / |
| Total proteins (g/L) | 71.1 (69.9-73.4) | 66.1 (55.6 -78.0) | 0.350 |
| Albumin (g/L) | 48.5 (46.7-51.6) | 40.6 (33.3-47.2) | <0.001 |
| Glucose (mmol/L) | 6.20 (5.72-6.95) | 6.50 (5.75-8.20) | 0.409 |
| Total cholesterol (mmol/L) | 6.0 (5.2-6.4) | 5.2 (4.0-5.8) | 0.124 |
| LDL-cholesterol (mmol/L) | 3.76 (2.93 -4.21) | 3.00 (1.93-3.46) | 0.091 |
| HDL-cholesterol (mmol/L) | 1.50 (1.41-1.65) | 1.55 (1.25-1.99) | 0.843 |
| Triglycerides (mmol/L) | 1.24 (0.77-1.45) | 1.33 (1.11-1.76) | 0.235 |
| AOPP (µmol/L) | 34.2 (22.7-43.6) | 54.7 (46.8-74.3) | <0.001 |
| TOS (µmol/L) | 7.4 (7.3-8.9) | 21.4 (12.7-31.4) | <0.001 |
| MDA (µmol/L) | 1.90 (1.56-1.92) | 1.51 (1.12-2.26) | 0.369 |
| PAB (U/L) | 75 (56-121) | 131 (87-154) | 0.010 |
| IMA (AU)*** | 0.094 (0.088-0.12) | 0.556 (0.502-0.722) | <0.001 |
| PON1 (U/L) | 249 (214-573) | 286 (239-793) | 0.316 |
| TAS (µmol/L) | 1512 (1017-1685) | 443 (297-651) | <0.001 |
| tSHG (mmol/L) | 0.526 (0.310-0.721) | 0.295 (0.225-0.358) | <0.001 |
| [25(OH)D] (nmol/L) ***** | NA**** | 11.0 (9.6-15.1) | / |
| hsCRP (mg/L) | 0.5 (0.35-1.45) | 10.8 (4.8-19.0) | <0.001 |
| SAA1 (mg/L) | 3.76 (2.69-7.91) | 8.82 (5.99-30.52) | 0.014 |
| sPD-L1 (ng/L) | 89.9 (51.8-107.1) | 297.5 (117.7 -545.9) | <0.001 |

*Chi-square test for categorical variables.
**The most common comorbidities in IPF patients were pulmonary hypertension (PH) (53.3%) and arterial hypertension (AH) (33.3%).
***AU – absorbance units; P from Mann-Whitney U test, $p$<0.05 is set as statistically significant.
AOPP - advanced oxidation protein products; TOS - total oxidative status; MDA – malondialdehyde; PAB - pro-oxidant-antioxidant balance; IMA - ischemia modified albumin; PON1 - Paraoxonase 1; TAS - total antioxidant status; tSHG – total sulfhydryl groups; [25(OH)D]-25-hydroxyvitamin D; hsCRP – high sensitivity C-reactive protein; SAA1 - serum amyloid A1; sPD-L1 - soluble programmed cell death-ligand 1.
**** NA- non- available for control group;
*****According to biochemical laboratory reference values for [25(OH)D]: Severe deficiency <25 nmol/L, deficiency 25-50 nmol/L, moderate deficiency 50-75 nmol/L, recommendation > 75 nmol/L.

Significantly higher sPD-L1 values in IPF patients compared to CG were found ($p$<0.001). [25(OH)D] concentration in the blood of IPF patients was in the area of deficient values (averagely lower than 30.0 nmol/L). We also found significantly higher hsCRP ($p$<0.001) and SAA1 proteins ($p$=0.014).

When comparing general biochemical parameters between IPF patients and CG, we could see that IPF patients had significantly lower albumin concentration ($p$<0.001), and lower LDL cholesterol, but with marginal significance ($p$=0.091). Other measured parameters were similar between the two study groups.

When comparing OS, inflammation markers and sPD-L1 levels according to the GAP stage of the disease, we could notice that PON1 activity and hsCRP level were lower, while tSHG and sPD-L1 were higher in IPF patients with more severe disease (i.e., II+III stage compared to I stage, $p$<0.05 for all) (Figure 1).

Given the remarkably interesting results for sPD-L1, we also performed multiple linear regression analysis to find predictors, i.e., parameters with the highest influence on variability in sPD-L1 concentration. The model consisted of parameters which initially showed significant influence: SAA1, PAB and albumin. The implemented backward method showed that the most influential parameter is PAB. This means that PAB showed about 38% of influence on sPD-L1 concentration increase (data not presented). Relationship between PAB and sPD-L1 concentration was direct, so patients with higher PAB levels tended to have higher sPD-L1 concentrations.

The association of respiratory function parameters and inflammation markers, OS, and [25(OH) D] in IPF patients was shown in Figure 2. It could be noticed that patients with forced vital capacity (FVC) less than 80% had significantly lower concentrations of AOPP and hsCRP, as well as lower levels of [25(OH)D] (Figure 2).

## Discussion

Our study finds that patients with IPF were upon advanced oxidative stress compared to

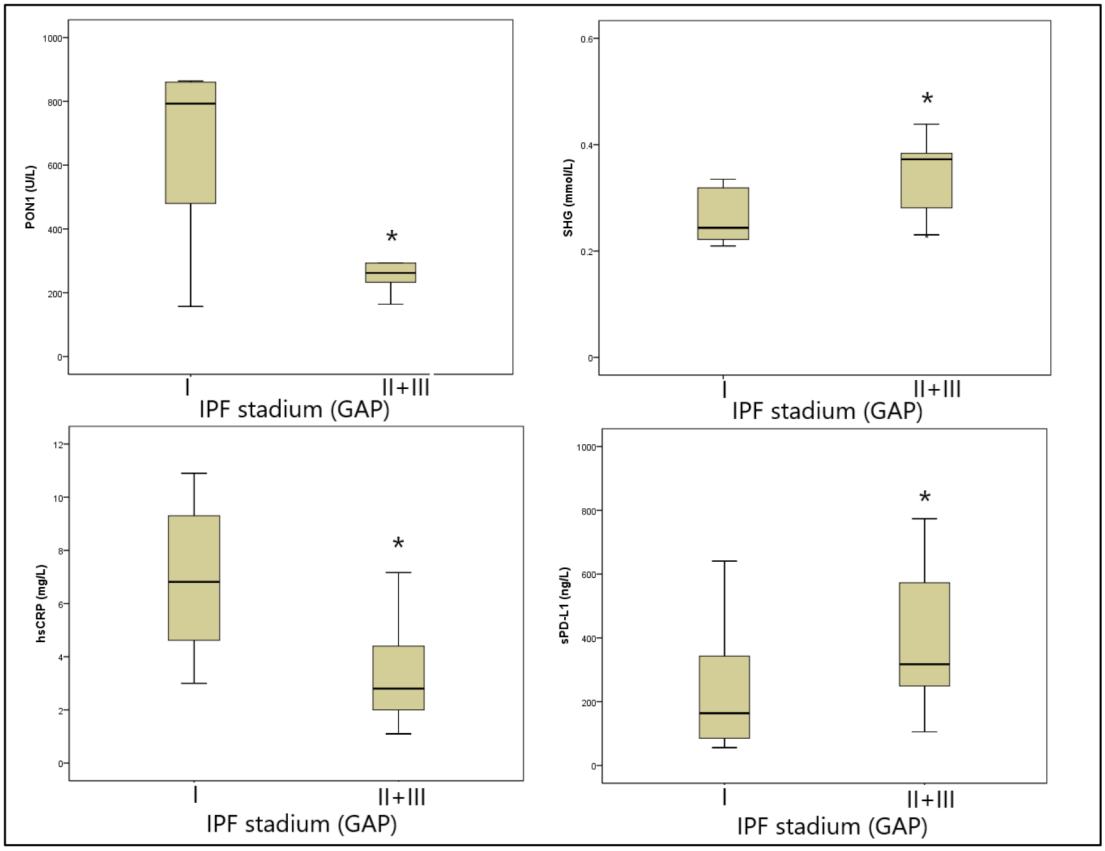


**Figure 1.** Redox status, inflammation and sPD-L1 according to GAP IPF stadia.

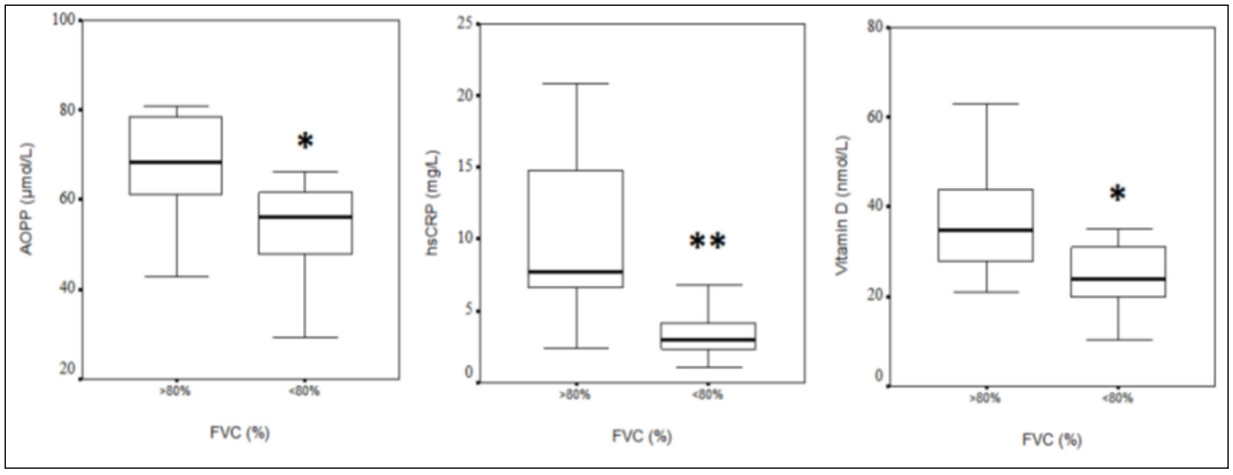


**Figure 2.** Association of respiratory function parameters and inflammation markers, OS and vitamin D in IPF patients.

healthy subjects, evidenced by significantly higher levels of AOPP, PAB, and TOS. Additionally, IPF patients had a lower level of antioxidative potency, reflected by the significantly lower TAS and tSHG, although PON1 activity was similar in both groups.

Furthermore, IMA was significantly higher in patients with IPF than in healthy subjects. This parameter is also a consequence of the oxidative stress existence which significantly contributes to the ischemic state in the systemic circulation. The cut-off value for the significant ischemia according to Barr-Or is 0.400 AU[7]. Our patients showed values higher than 0.500 AU which is a sign of significant chronic ischemia.

We have also shown lower PON1 activity and hsCRP levels, whereas higher tSHG and sPD-L1 in patients with a more severe stadium of the disease (II+III stage compared to I). Our results of increased OS in those patients are in line with the study of Ugurlu et al[22]. They showed that TOS and oxidative stress index were higher in patients with IPF, although TAS values were similar to those in control group.

Taking all together, we could suppose that patients with IPF are in a state of pronounced oxidative stress which may at least in part be related to the inflammatory nature of their disease.

Daniil et al[23] showed that total hydroperoxides were significantly higher in IPF patients than in controls. Also, it is interesting that levels of hydroperoxides in sera of those patients were positively correlated with the degree of dyspnoea and negatively correlated with FVC. Similarly, several years later Matsuzawa et al[24] found significantly higher levels of serum hydroperoxide in patients with IPF, as compared with controls. Furthermore, serum hydroperoxide concentrations in patients with acute exacerbation were significantly higher than those patients with stable disease. The authors concluded that, since hydroperoxides in IPF were significantly correlated with lower FVC and acute exacerbation, oxidative stress may be involved not only in IPF development but also in its progression and suggested that hydroperoxides can be a biomarker for the clinical diagnosis in IPF.

Muramatsu et al[25] in their retrospective study evaluated the effect of antioxidants, such as inhaled N-acetylcysteine (NAC) monotherapy (i.e., acts as scavenger and restores glutathione), on redox balance and lung function in patients with early untreated IPF. Baseline total glutathione (GSH) concentrations were significantly lower and oxidized glutathione concentrations (GSSG) were significantly higher in IPF patients than in controls. Veith et al[26] also measured GSH concentrations which were lower in IPF patients for 50% in comparison with CG, but GSSG values did not significantly differ between these groups. However, there is still a lot of controversy about its use in the treatment of IPF[7,27].

According to other authors' discoveries[28], some antioxidants can play a significant role and should be considered as a potential treatment for IPF, but definitely, it is necessary to conduct new investigations on different antioxidants use efficacy and safety as mono- or add on therapy.

Data related to vitamin D concentration and its role in IPF pathogenesis and prognosis are extremely poor. In our study, concentration of

[25(OH)D] in IPF patients' blood was in the area of severe deficient values. Tzilas et al[29] found that there was no significant difference in vitamin D serum concentration between IPF and non-IPF samples, but vitamin D levels were correlated with disease severity, as assessed by FVC and were predictive for mortality.

Olson et al[30] found that in the IPF population the highest mortality was in the winter months, suggesting that there is a significant impact of vitamin D level (i.e., decreased in winter) on it. Shi et al[31] reported that chronic vitamin D deficiency may induce lung fibrosis, through renin-angiotensin system activation, which subsequently stimulates the expression of pro-fibrotic factors and activates the fibrotic cascade. Zhang et al[32] reported that early vitamin D supplementation significantly reduced the severity of pulmonary fibrosis and inflammatory cell accumulation in the bleomycin-induced pulmonary fibrosis in mouse model on supplementary days 14, 21, and 28.

In our study, we have also found significantly increased hsCRP and SAA1 proteins in IPF patients compared to CG, which are positive reactants of the acute phase response of the body. Lee et al[33] reported that increased levels of CRP were significantly associated with poor survival in IPF patients.

Literature data about the role of SAA in IPF are scarce[34,35]. Researches showed that liver synthesizes SAA protein which can stimulate the production of various cytokines, thus playing an important role in acute immune response[36]. We have recently shown significantly higher SAA1 levels in newly diagnosed lung cancer patients *vs.* healthy control group[37] and assumed that SAA1 can be a good predictor of patients' survival and can be a biomarker of disease severity.

Our current study has found that serum concentrations of sPD-L1 were higher in IPF patients compared with CG. This finding could suggest some similarities in the biology of IPF and lung cancer since we have previously also shown its increased levels in patients with lung cancer[37].

Also, we have performed multiple linear regression analysis to find predictors, i.e., parameters with the highest influence on variability in sPD-L1 concentration. The model consisted of parameters that initially showed significant influence: SAA1, PAB, and albumin. The implemented backward method showed that the most influential parameter, i.e. PAB, showed about 38% of the influence on sPD-L1 concentration increase. Patients with higher PAB levels tended to have also higher sPD-L1 concentrations. This could be explained by disbalance between pro-oxidants and anti-oxidants in IPF patients, as reflected with PAB and the general disturbance of the immune system seen in those patients that goes along with impairment mirrored in sPD-L1 increase. Accumulated data at present indicate that sPD-L1, which can be easily estimated in clinical practice, may have an especially important role in immunopathogenesis and the potential to reflect treatment responses in IPF and some other autoimmune disorders, as well. It may be speculated that sPD-L1 might be a new biomarker with a significant impact on potential treatment strategies including immunotherapy in a variety of diseases[9,37]. Our above-mentioned results require further investigations in a larger patient's population.

### *Limitations*

The limitations of our current study should be reported. Namely, due to the small sample size, new studies with a larger number of participants are required to validate our results. However, the strength of the current study lies in the fact that we have investigated several novel biomarkers in patients with IPF which were not mutually investigated before. These results might add a significant contribution to better recognition of pathophysiological mechanisms of IPF.

## Conclusions

Based on the presented results we might suppose that IPF patients are in a state of profound oxidative stress compared to healthy people, which is at least in part related to the inflammatory component of the disease. The depth of redox imbalance evidenced in our study is related to the IPF stage. IPF patients exhibited significantly higher concentrations of inflammation markers (i.e., hsCRP and SAA1) compared to healthy people. They also exhibited a significant deficiency of [25(OH)D], so its substitution might be regarded as a potential treatment option for IPF, also. Moreover, sPD-L1 may have significant role in immune responses in IPF. In addition, according to our research, antioxidants might be considered as a potential treatment option, at least for some subpopulations of IPF patients. Further investigations in a larger patient's population are necessary to validate our results and to propose potential treatment strategies including immunotherapy in IPF.

### Conflicts of Interest

The authors declare no conflicts of interest.

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