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PROOXIDANT-ANTIOXIDANT BALANCE, ADVANCED OXIDATION PROTEIN PRODUCTS AND LIPID PEROXIDATION IN SERBIAN PATIENTS WITH PARKINSON'S DISEASE

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Running title: Oxidative stress in patients with Parkinson's disease.

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Abstract

Background. Biomarkers of oxidative stress are relevant in the evaluation of the disease status and prooxidant-antioxidant balance, advanced oxidation protein products and lipid peroxidation products (malondialdehyde and 4-hydroxynonenal) are being extensively evaluated regarding their relationship with clinical presentation and disease severity.

The aim of study was to evaluate the levels of above mentioned parameters in plasma of 39 men and 17 women with Parkinson's disease, originated from the Republic of Serbia and their relation to clinicopathological characteristics (gender, age at examination, duration of the disease, and Hoehn and Yahr score) and oxidative status.

Results The incidence of disease was 2:1 towards males. The investigated oxidative parameters were gender and Hoehn and Yahr related. Significant association of higher Hoehn and Yahr scores was observed for malondialdehyde ($p = 0.01$) and prooxidant/antioxidant balance ($p = 0.02$). Relation between oxidant/antioxidant status was further supported by observed positive correlation between 4-hydroxynonenal ($p = 0.04$) and prooxidant-antioxidant balance ($p = 0.03$). Finally, the multivariate analysis indicated that prooxidant/antioxidant balance and malondialdehyde were partially determined

by gender (10.6% and 7.6%) and Hoehn and Yahr scores (13.6% and 18.8%), while Hoehn and Yahr scores contributed to the variance of advanced oxidation protein products with 13.2%.

Conclusion. Our results indicate the higher level of oxidative stress (oxidant/antioxidant imbalance) and possible relation of several markers with gender and disease stage in patients with Parkinson's disease The analyzed markers could be used to specify the severity of oxidative stress; however their potential value should be analyzed in further studies.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting approximately 1 percent of the worldwide population older than 55 years. According to recent meta-analysis, an overall prevalence of PD is 315 per 100 000 individuals, which intensifies gradually with age, increasing from 428 per 100 000 in the age group 60-69 years, to 1903 per 100 000 individuals in the group aged 80 years and older [1]. Statistically, it is more frequent in males (65.5 per 100 000 person-years) than females (36.5 per 100 000 person-years) [2]. The average mortality rate of patients with PD is 2 per 100 000 individuals, while in Republic of Serbia it is 3.43 per 100 000 citizens, being one of the highest in Europe [3].

This gradual but progressive chronic condition is manifested by cardinal motor features such as rigidity, bradykinesia, postural instability, and tremor as well as by non-motor symptoms that include constipation, autonomic dysfunction, neuropsychiatric problems, and sensory and sleep difficulties [4]. In general, these symptoms usually develop as a result of degeneration and selective loss of dopaminergic neurons in *substantia nigra pars compacta* (SNpc), dopamine deficiency in *corpus striatum* and formation of intraneuronal inclusions of protein α-synuclein (named Lewy bodies) [5] in mid-brain, brain stem and olfactory bulb.

Although PD was first described two centuries ago, the exact etiopathophysiological mechanisms of disease are still a matter of controversy. Among various putative factors that may contribute to PD pathogenesis, the neuroinflammation and oxidative damage may be the pivotal players. They could trigger the cascade of events leading to lipid, protein and DNA damage, cellular dysfunction and eventual cell death. Indeed, at cellular level, PD onset and progression might be associated with excessive generation of reactive oxygen/nitrogen species (ROS/RNS), modifications in catecholamine metabolism and antioxidant (AO) protective systems, augmentation of iron deposition in SNpc [6] and with higher expression/increased levels of inflammatory mediators (cytokines and chemokines) [5].

Products of lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) might be suitable biomarkers for several diseases [7, 8] since they can propagate and amplify oxidative injury. MDA is a reactive aldehyde generated by degradation of arachidonic acid and larger polyunsaturated fatty acids (PUFAs), while 4-HNE is produced as a major ω-6 polyunsaturated fatty acids peroxidative decomposition and possesses cytotoxic, hepatotoxic, mutagenic, and genotoxic properties [9]. MDA appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most toxic [10].

Beside lipids, proteins are one of the main targets of ROS. Advanced oxidation protein products (AOPPs) [11] are used as a degree of oxidative stress's protein damage in several pathological conditions like uremia and chronic renal failure [12, 13] as well as different neurological pathologies, such as multiple sclerosis [14], ALS [15], mitochondrial myopathies [16], etc. Additional candidate for PD biomarker is prooxidant-antioxidant balance (PAB), a state of dynamic balance generated under conditions of homoeostasis between free radicals that are produced and those utilized (scavenged) [17].

Since PAB, level of AOPP and lipid peroxidation end products (MDA and 4-HNE) might be tracers of oxidative stress and important in the pathogenesis of several neurodegenerative disorders, current study aims to estimate their levels in plasma of patients with PD and their correlation with clinicopathological features (gender, age at examination, duration of the disease, and Hoehn and Yahr staging).

Materials and methods

Patients

The study comprised 39 men and 17 women with idiopathic PD, originated from the Republic of Serbia, aged 35-82 years. Patients were diagnosed according to the UK Parkinson's Disease Society Brain Bank Research criteria, UK, London [18] and staged according to H&Y score [19] by neurologist experienced in movement disorders. All patients were on L-dopa medications. None of the patients in this study was diagnosed with the H&Y score 5. Subjects with cancer history, marked autonomic disturbances, chronic renal, inflammatory, liver, and hematological diseases and those on antipsychotic drugs were excluded from the study.

Blood samples were collected at the Clinic of Neurology, Medical Faculty of Medical Military Academy, University of Defense in Belgrade, Republic of Serbia, Belgrade, while all analysis were approved by the local Ethics Committee according to the Declaration of Helsinki (1975). All participants provided written informed consent.

Biochemical measurements

Peripheral blood samples were collected into Vacutainer® (BD Diagnostics, Plymouth, UK) plastic tubes with K$_2$EDTA anticoagulant and centrifuged at 1500 g, for 10 min, at 4°C, within 30 min of collection. Plasma was carefully separated and stored at -80°C until further processing.

4-hydroxynonenal

The concentration of 4-HNE was determined by enzyme-linked immunosorbent assay for quantitative determination in human samples (Human HNE ELISA Kit, MyBioSource, California, USA). Aliquots of standards and plasma samples (50 µl) were added to a microtiter plate and processed according to the manufacturer instructions. Concentration of 4-HNE was determined spectrophotometrically (LKB Vertriebs GmbH, DV 990 BV4-6, Austria) at 450 nm, by comparing optical density (OD) of a sample to the standard curve. The standards and plasma samples were measured in one assay while differences between parallels were less than 6 %. The 4-HNE levels are given as µg/ml.

Malondialdehyde

Plasma MDA concentration was monitored using TBARS assay according to [20]. Aliquots of blank (0.05 M TRIS HCl, pH 7.4), MDA standards (range of 1-10 µmol/l) and plasma samples (300 µl) were added into 600 µl of TBA-containing reagent tubes. TBA containing reagent is made from TCA (trichloroacetic acid), TBA and HCl. After vortexing, heating for 5 min in a boiling water bath and cooling, they were centrifuged at 1000 g, for 10 min, at 4°C. The absorbance was measured on microplate reader at 535 nm and MDA levels are expressed as µmol/l.

Advanced oxidation protein products

AOPP levels were estimated according to Witko-Sarsat method [12]. Briefly, 400 µl of plasma sample was diluted in PBS (1:5) while for the standard curve, 20 µl of potassium iodide was added to 400 µl of chloramine-T solution. The reaction was stopped with 40 µl of acetic acid and the absorbance of the reaction mixture was measured in a microplate reader at 340 nm. AOPP concentrations are expressed as µmol/l of chloramine-T equivalents.

Prooxidant/antioxidant balance

Evaluation of PAB was performed according to the method by Almandari and coworkers [21]. Following the incubation for 2 min at room temperature in dark, 200 µl of working solution (1 ml TMB cation solution with 10 ml TMB solution) was added to a 96-well microtiter plate and, in each well,

mixed with either 10 μl of each plasma sample, standard or blank (dH$_2$O), incubated in a dark place for 12 min, at 37°C and, then the reaction was stopped by adding 100 μl of 2 N HCl. According to the percentage of hydrogen peroxide in the standard solution, the values of PAB in plasma samples were determined with microplate reader at 450 nm, with a reference wavelength of 620 or 570 nm, by comparing OD of a sample to the standard curve. PAB values are expresses in arbitrary units (HK).

Statistical analysis

The statistical analyses were performed using the GraphPad Prism and IBM SPSS 23.0 software. Data are expressed as mean ± SD. The normality of the distribution was tested by Shapiro-Wilks test. Since examined variables had non-Gausian distribution and based on the obtained median and percentile values, the variables cohort was divided into tertiles (subgroups with low, middle and high concentration) as previously reported [22]. Kruskal-Wallis test was used to test the differences among tertiles. To analyze the association of examined variables with demographic and clinical characteristics, the chi-square test was used. The Spearman univariate correlation was used the test the relation between AO/oxidant parameters, as well as between H&Y score/AO or oxidant variables. The general linear model (GLM) was used to test the univariate and multivariate analysis. The p value < 0.05 was considered statistically significant.

Results

In current study, the average age of patients was 64.70 ± 8.29 years, and the majority was men (69.64 %), while H&Y scores 2 and 3 were almost equally represented (35.71 %, 32.14 %, respectively) (Table 1).

The associations of 4-HNE, MDA, PAB, and AOPP values with clinicopathological characteristics are summarized in Table 2. For all investigated parameters, a significant difference among tertile subgroups was detected (4-HNE, H = 48.22; MDA, H = 44.59; AOPP, H = 47.39; PAB, H = 46.94; p < 0.001, Kruskal-Wallis).

There was a significant gender- difference in proportions of 4-HNE (χ2 p = 0.02), MDA (χ2 p = 0.04), and PAB (χ2 p = 0.02) tertiles, while the association of the examined variables with H&Y score was only observed for MDA (χ2 p = 0.01) and PAB (χ2 p = 0.02) tertiles (Table 2).

Middle range values of MDA concentration were more frequent in H&Y score 2 (51.61 %), while high values (> 0.89µg/ml) were found in 71.43 % of patients with H&Y score 3. On the other hand, the middle range PAB concentrations were observed in 37.04 % of subjects with H&Y score 2, whereas 57.14 % of patients exhibited low PAB levels (< 9.46 HKU) in H&Y score 3 (Table 2). Although a significant association was not found between PAB, oxidative parameters (4-HNE, MDA and AOPP) and age/disease duration factors, elevated concentrations of the examined parameters were more frequent in patients aged 59 - 70 years than in any other age group, and among those with disease duration from 3 - 8.5 years (Table 2).

Univariate correlation analysis between oxidant and AO parameters indicated that PAB status was positively correlated with 4-HNE concentration (Figure 1 (a)), whereas there was no significant correlation between PAB and other oxidative parameters, such as MDA and AOPP (Figure 1 (b), (c) respectively).

The examination of relation between clinicopathological parameters and oxidant/AO status revealed a significant positive correlation (p = 0.02) between age and MDA level (Figure 2 (c)) as well as between H&Y score and AOPP concentration (p = 0.03, Figure 3 (d)). There was no significant correlation between disease duration and PAB (r = - 0.04, p = 0.98), 4-HNE (r = 0.13, p = 0.33), MDA (p = - 0.17, p = 0.20), and AOPP (r = 0.04, p = 0.76) levels.

Finally, the GLM univariate analysis of individual associations between clinical and biochemical parameters is summarized in Table 3, with *eta* values as a measure of the tightness of the association and explained variability. Multivariate analysis showed that gender is a significant variance component of PAB (F=5.587, p=0.022, eta 10.6%) and almost shows the statistical significance of association with MDA scores (F=3.840, p=0.056, eta 7.6%). H&Y score significantly effects MDA (F=3.628, p=0.020, eta 18.8%), while significant portions of the variance of PAB and AOPP (eta 13.6%, 13.2%, respectively) were near the borderline significance (F=2.460, p=0.074; F=2.382, p=0.08, respectively). Other associations were not significant.

Discussion

The results of this study revealed that patients could be divided into cohorts according to different levels of investigated parameters. In general, these parameters were gender-, age- and H&Y score-

related. Literature highlights that PD is gender- and age-related disorder, as males and elder individuals are more predisposed [23, 24]. Since later PD development in women could be associated with fertile life span, it is assumed that female sex hormones have beneficial effect and might postpone PD onset and progression [25]. In fact, the data presented herein are in accordance with those findings given that the incidence of PD was 2:1 towards males along with later onset of 4.43 years in female patients than in males, contributing to differences between investigated groups.

Small amounts of ROS/RNS, are necessary for normal functioning of the cell since they act as important second messengers. However, their overproduction and long term exposure is linked to malfunction of AO defense mechanisms and imbalance between oxidant/AO processes, inducing a progressive damage of DNA, proteins and lipids. PUFAs in membrane lipids are one of the main targets of ROS/RNS, whose peroxidation enable the generation of lipid hydroperoxides, like MDA and 4-HNE. Although MDA is more chemically stable and less toxic than 4-HNE, its biological function and possible dual role are poorly understood. Just a few recent publications indicated that MDA may act as signaling messenger and regulator of gene expression and, thus, be potentially therapeutically valuable. However, most studies are focused on its detrimental effects since it is believed that MDA is excessively formed under stress conditions and has high capability to react with various biomolecules, resulting in their biochemical alterations and massive accumulation, as observed in different pathological states [10]. For instance, increase of MDA content in cerebrospinal fluid was found in drug naive patients with PD [26]. In regard to the 4-HNE concentration, it is estimated at 0.05-0.15 mM in human blood and serum, whereas in pathological conditions it is elevated (more than 100 mM) [27]. The high 4-HNE toxicity might be explained by the post-translational modifications [7] of its downstream effectors such as proteins involved in the proteasome system, leading to system's failure and neuronal cell death [28]. Besides MDA and 4-HNE, modulation of PAB level, a surrogate marker of factors that promotes and controls systemic oxidative stress, should be considered serious as the prolonged production of toxic species is related to oxidative stress-induced cell/tissue injuries, ultimately resulting in clinical diseases.

In current study, a gender-related association of MDA, 4-HNE and PAB plasma levels were implied, stressing the presence of potential biochemical basis for the epidemiologic differences in the disease susceptibility between male and female patients with PD. Further, a significant association of higher H&Y scores and elevated MDA level was obtained. This finding might indicate the higher level of oxidative stress (oxidant/antioxidant imbalance) and suggest that MDA could be used to indicate the severity of oxidative stress in patients with PD. Finally, the relation between oxidant/antioxidant status

in patients with PD is further supported by the observed correlation between 4-HNE levels and PAB levels.

No significant correlation between the disease's stage and lipid peroxidation parameters was detected most likely due to the fact that subjects involved in this study were in the different stages of disease. This finding is consistent with the study of Agil and coworkers [29], who reported the lack of the same correlation given that clinical symptoms appear when PD is in advanced stage and approximately 80 % of dopaminergic neurons in SNpc are already dead.

The correlation between the clinicopathological characteristics of patients and oxidative parameters was shown to be positive between age and MDA level. The possible contribution of aging in the pathogenesis of PD is suggested by disease occurrence in late middle age and increased prevalence at older ages [30]. The observed accumulated level of lipid peroxides in elder patients with PD might be a result of prolonged exposure to overabundant free radicals derived from some endogenous or exogenous neurotoxic species that initiate coordinated reduction of PUFA content, as a substrate available for lipid peroxidation, and augmentation of MDA levels.

Besides lipids, other molecular targets of ROS are proteins, the most abundant biomolecules in the organism, whose oxidation generates AOPP [11]. Although estimation of their levels is employed in monitoring of oxidative-stress-induced damage to proteins in several neurodegenerative disorders, like mitochondrial myopathies or multiple sclerosis and amyotrophic lateral sclerosis [14], there is a scarce data about their enrollment in PD onset and its progression [31]. Just recently, Medeiros and coworkers [31] showed that AOPP level was significantly increased in patients with PD and proposed that it could be used as a potential marker for evaluating the protein damage, which is linked to oxidative stress in PD. In this study, a positive correlation between AOPP plasma level and H&Y score was established. The increased AOPP level indicates a more profound oxidative damage to proteins and intensive inflammatory cascade, which in turn causes a higher level of nervous tissue damage. This might result in a more severe clinical picture of inflammation under conditions of disrupted defense potentials of the organism [32] in advanced stages of PD.

Finally, some issues need to be addressed, since present study has some limitations. There is a possibility that selection bias influenced the significance of our findings. Further research using the larger sample size and homogenous (gender, age, years since diagnosis) cohort is needed to confirm our

results. Nevertheless, it provides the indications for the correlation between peripheral parameters of oxidative stress and clinicopathological features in PD patients.

Conclusions

In conclusion, our results show that several markers of oxidative stress in blood plasma are possibly related to gender and PD stage. We observed a correlation of gender with HNE, MDA and PAB that was further supported by GLM analysis regarding MDA and PAB level. In addition, H&Y score determined a significant portion of MDA variance and possibly the PAB and AOPP level. Whether the examined parameters in this study could be used as indicators of oxidative stress in patients with PD and severity of disease requires further research.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

JM performed experiments, acquisited data, suggested the methodology for their analyses and drafted the manuscript. DD participated in the interpretation of obtained results and writing the manuscript whereas SP contributed to analysis of data and drafting of the manuscript. AS and MM assisted during biochemical measurements. MP, TVI and MS designed and supervised experimental setup as well as edited the manuscript. TVI also selected the patients for the study. All authors approved the final version of the manuscript.

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Legends to Figures

Figure 1.   Correlation between PAB and (a) 4-HNE, (b) MDA and (c) AOPP levels in plasma of patients with PD.

Figure 2.   Correlation between age and (a) PAB, (b) 4-HNE, (c) MDA and (d) AOPP levels in plasma of patients with PD.

Figure 3.   Correlation between H&Y score and (a) PAB, (b) HNE, (c) MDA and (d) AOPP levels in plasma of patients with PD.
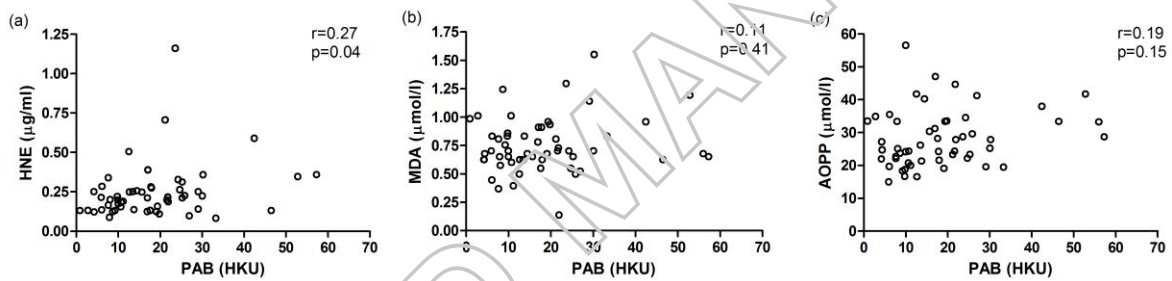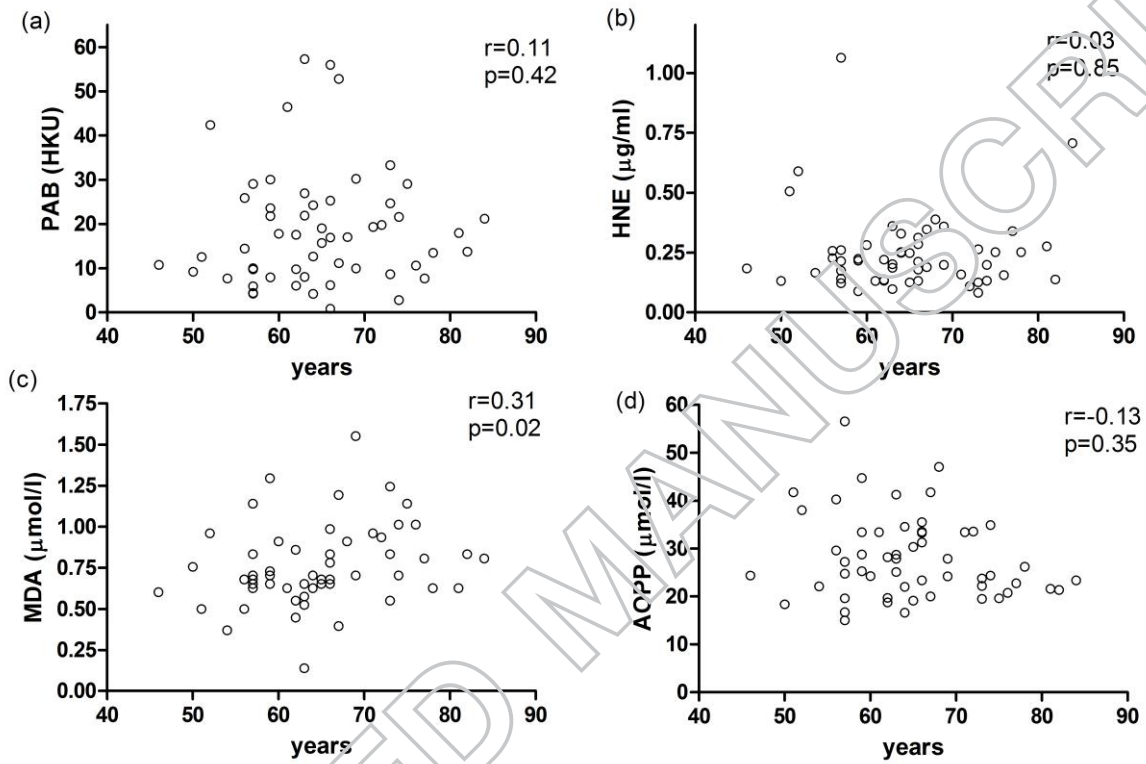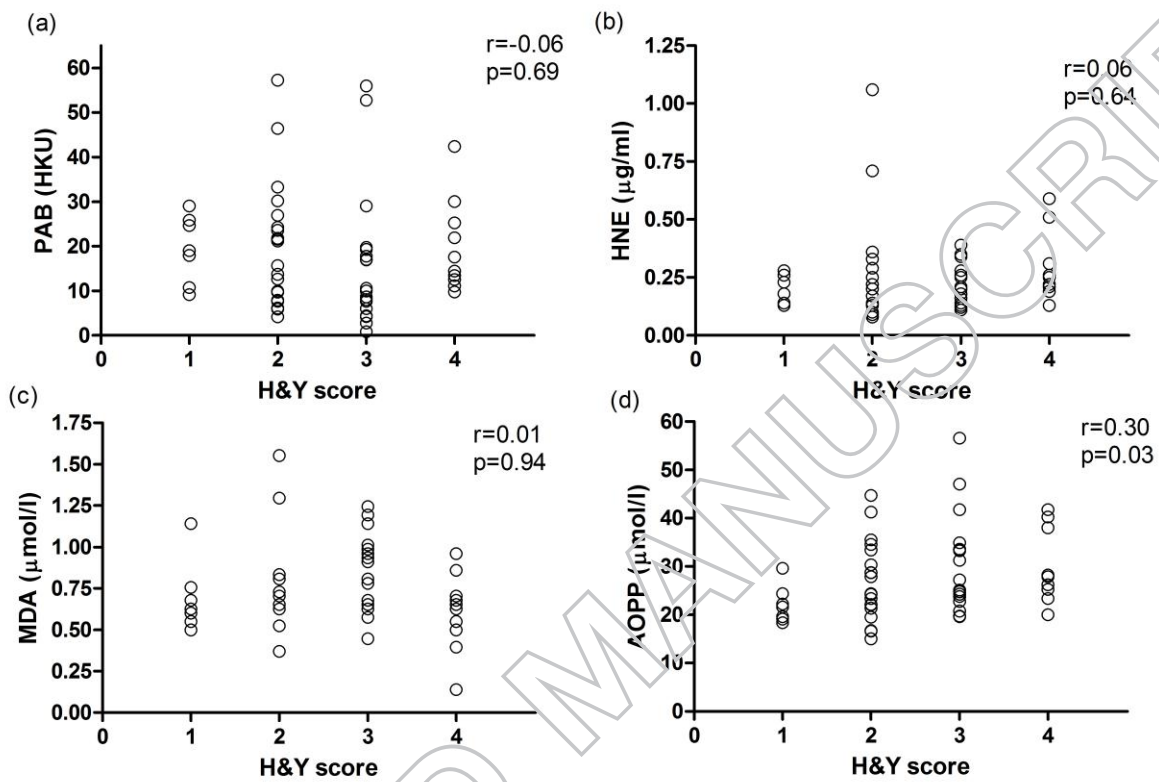
Figure 1:

Figure 2:

Figure 3:

Graphical Abstract:
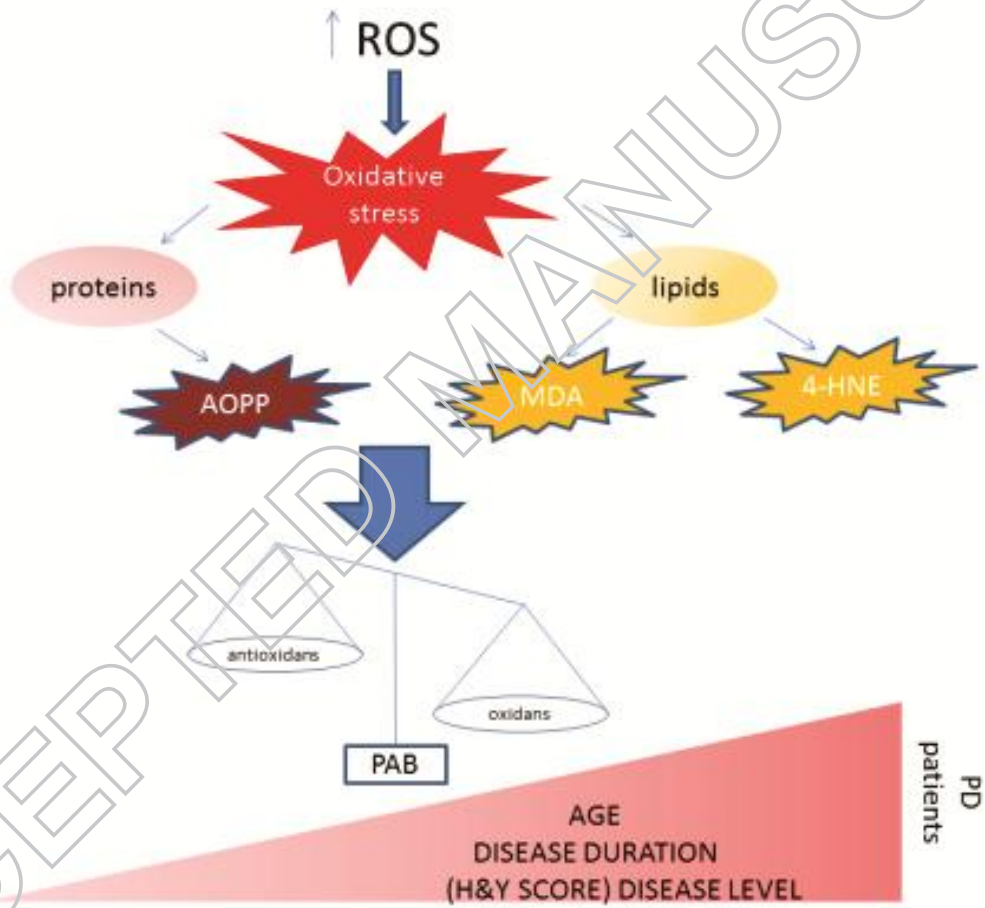
Table 1. Demographic and clinical data of patients with PD

Gender, N (%)	
Male	39 (69.64)
Female	17 (30.36)
Age at examination (years)	64.70 ± 8.29
Age at disease onset (years)	57.68 ± 9.78
Disease duration (years)	6.43 ± 4.25
H&Y score, N (%)	
1	7 (12.50)
2	20 (35.71)
3	18 (32.14)
4	11 (19.64)
5	0

Values are mean ± SD

N - number of patients, (%) - fractions in relation to N

Table 2. 4-HNE, MDA, PAB and AOPP levels in plasma of patients with PD, in relation to clinicopathological characteristics

Factors		HNE (µg/mL)				MDA (µmol/L)				PAB (HKU)			
		<0.14	0.14-0.28	>0.28	p	<0.63	0.63-0.89	>0.89	p	<9.46	9.46-24.96	>24.96	p
	N	14.00	19.00	23.00		11.00	31.00	14.00		14.00	27.00	15.00	
	mean ± SD	0.12±0.02	0.19±0.03	0.41±0.25		0.47±0.13	0.71±0.08	1.09±0.18		5.98±2.42	16.08±4.67	35.63±11.95	
Gender (N, %)	Male	9 (64.29)	17 (89.47)	13 (56.52)	0.02*	9 (81.82)	23 (74.19)	7 (50)	0.04*	12 (85.71)	20 (74.07)	7 (46.67)	0.0?
	Female	5 (35.71)	2 (10.53)	10 (43.48)		2 (18.18)	8 (25.81)	7 (50)		2 (14.29)	7 (25.93)	8 (53.33)	
Age at examination (N, years, %)	<59	2 (14.29)	6 (31.58)	5 (21.74)		4 (36.36)	7 (22.58)	2 (14.29)		5 (35.71)	5 (18.52)	3 (20)	
	59-70	7 (50)	10 (52.63)	12 (52.17)	0.67	6 (54.55)	17 (54.84)	6 (42.86)	0.33	6 (42.86)	14 (51.85)	9 (60)	0.7
	>70	5 (35.71)	3 (15.79)	6 (26.09)		1 (9.09)	7 (22.58)	6 (42.86)		3 (21.43)	8 (29.63)	3 (20)	
Disease duration (N, years, %)	<3	3 (21.43)	3 (15.79)	3 (13.04)		2 (18.18)	5 (16.13)	2 (14.29)		3 (21.43)	5 (18.52)	1 (6.67)	
	3-8.5	9 (64.29)	10 (52.63)	14 (60.87)	0.81	5 (45.45)	17 (54.84)	11 (78.57)	0.41	8 (57.14)	16 (59.26)	9 (60)	0.7
	>8.5	2 (14.29)	6 (31.58)	6 (26.09)		4 (36.36)	9 (29.03)	1 (7.14)		3 (21.43)	6 (22.22)	5 (33.33)	
H&Y score (N, %)	1	2 (14.28)	3 (15.79)	2 (8.7)		3 (27.27)	3 (9.68)	1 (7.14)		1 (7.14)	3 (11.11)	3 (20)	
	2	5 (35.71)	5 (26.32)	10 (43.48)	0.72	2 (18.18)	16 (51.61)	2 (14.29)	0.01*	5 (35.71)	10 (37.04)	5 (33.33)	0.0?
	3	6 (42.86)	6 (31.58)	6 (26.09)		2 (18.18)	6 (19.54)	10 (71.43)		8 (57.14)	7 (25.93)	3 (20)	
	4	1 (7.14)	5 (26.32)	5 (21.74)		4 (36.36)	6 (19.54)	1 (7.14)		0.00	7 (25.93)	4 (26.67)	

* statistically significant, N - number of patients, (%) - fractions in relation to N, p - statistical values

Table 3. GLM univariate analysis of the associations between clinical and biochemical parameters.

		MDA	HNE	PAB	AOPP
Gender					
	F	2.730	0.226	4.122	1.638
	p	0.106	0.637	0.049	0.207
	eta	6%	0.5%	8.7%	3.7%
Age of	F	0.029	0.277	0.526	2.347
examination	p	0.972	0.759	0.595	0.108
	eta	0.1%	1.3%	2.4%	9.8%
H&Y	F	2.956	0.650	1.633	3.771
	p	0.043	0.587	0.196	0.017
	eta	17.1%	4.3%	10.2%	20.8%
Disease	F	0.223	0.113	0.039	1.019
duration	p	0.801	0.893	0.962	0.369
	eta	1%	0.5%	0.2%	4.5%

MDA - Malondialdehyde, HNE - 4-hydroxynonenal, PAB – Prooxidant/antioxidant balance, AOPP- Advanced oxidation protein products, Eta-quantified variance components, p<0.05 indicates statistical significance.