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# Levels of selected oxidative stress markers in the vitreous and serum of diabetic retinopathy patients

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**Purpose:** In diabetes, an impaired antioxidant defense system contributes to the development of diabetic retinopathy. The main objective of this paper was to find correlations of oxidative stress parameters within and between the vitreous and serum in patients with type 2 diabetes who had developed proliferative diabetic retinopathy.

**Methods:** The study included and compared two groups of patients who underwent vitrectomy: 37 patients with type 2 diabetes and proliferative retinopathy (PDR), and 50 patients with non-diabetic eye disorders (NDED). Vascular endothelial growth factor (VEGF), advanced oxidized protein product (AOPP), and oxidative stress markers (direct lipid hydroperoxidation (LPO), malondialdehyde (MDA), total superoxide dismutase (SOD), and glutathione (GSH)) were measured in the vitreous and serum of both groups and correlated with one another, between humoral compartments and with gender, age, and serum glucose levels.

**Results:** In the vitreous of PDR patients, VEGF, LPO, and MDA ( $p < 0.05$ ) were increased and SOD values were slightly lowered ( $p < 0.05$ ) than in NDED patients. Vitreous AOPP and GSH showed no differences between the groups. In the serum, AOPP, MDA, and SOD were increased ( $p < 0.05$ ) and VEGF was slightly increased ( $p < 0.05$ ) in the PDR group compared to NDED. With regard to gender, similar changes were recorded for both groups, except for the lower serum MDA in males than females in the NDED group. Advanced age showed no significant effect on changes of measured parameters in the vitreous. In the serum, VEGF was positively correlated ( $p < 0.05$ ) and MDA and SOD negatively correlated ( $p < 0.05$ ) with increasing age. Among measured parameters within and between the vitreous and serum, several correlative links occurred in the PDR group that were not present in the NDED group. The most prominent correlation changes were between serum LPO and vitreal LPO, serum SOD and vitreal LPO, serum LPO and serum SOD, and vitreal VEGF and serum SOD.

**Conclusions:** Among the selected oxidative stress markers, SOD and LPO were highly correlative in both the vitreous and serum in PDR compared to patients without metabolic disorders. Their correlations suggested that monitoring their mutual alterations might be informative during PDR development and should be considered in further research.

In 2012, more than 371 million people worldwide had diabetes. This figure continues to increase, and it is estimated that many cases remain undiagnosed [1]. Hyperglycemia-induced oxidative stress is considered to be a link between elevated glucose and other metabolic abnormalities important in the development of diabetic complications of neuropathy, nephropathy, myocardial injury, and retinopathy [2,3].

Diabetic retinopathy is a serious microvascular complication that affects the retina, with symptoms appearing late after the onset of diabetes. Changes are reflected in the structure and function of retinal capillaries, arterioles, and venules as a result of pathological processes, such as the

formation of microvascular obstructions. This causes retinal ischemia, neovascularization, and abnormal microvascular permeability, which leads to the formation of retinal edema. Ultimately, both mechanisms may cause retinal detachment and blindness [4-7].

Retinal expression of the angiogenesis inducer vascular endothelial growth factor (VEGF) is implicated as a mediator of non-proliferative and an initiator of proliferative diabetic retinopathies. Oxidative stress mediates the hyperglycemia-induced pathological effects of VEGF on the microvascular complications of diabetes, and increases the permeability of the blood-retinal barrier [8]. In experimentally induced diabetes, the expression and activity of the antioxidant defense system changed along with the transcriptional factors and regulatory proteins involved in antioxidant defense [9-14]. Analyses of concomitant changes of the oxidative stress markers LPO, MDA, SOD, and GSH, as well as other

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markers, such as VEGF or AOPP, can be found in the literature, individually presented in different studies, assayed by different methods on a different number of examined subjects, thus preventing the examination of possible correlations and ties between all of the assayed parameters in both physiologic fluids from the same patients [15-20].

In this study, VEGF, AOPP, LPO, MDA, SOD, and GSH were measured in two physiologic media, vitreous and serum, in a study group consisting of type 2 diabetes patients with developed proliferative diabetic retinopathy (PDR), compared to patients without metabolic disorders. The objective of this study was to depict concomitant models of change of significant high positive or negative physiologic correlations among these markers, within and between two physiologic media, vitreous and serum, that could be of practical importance for further research or monitoring.

## METHODS

*Participants in the study:* The study included two groups of patients who underwent vitrectomy during the study period. The study group consisted of patients with type 2 diabetes with proliferative diabetic retinopathy—the PDR group (n=37, average±SD age=68.90±11.65)—and it was compared to the second group, which consisted of patients with non-diabetic eye disorders—the NDED group (n=50, average±SD age=61.24±11.94)—having anatomic vitreoretinal disorders (macular hole, retinal detachment, epiretinal membrane). There were no statistically significant differences between mean demographic parameters, age range, or sex of the two groups. The diagnosis of type 2 diabetes was based on World Health Organization criteria [21], and proliferative diabetic retinopathy was classified according to the modified Airlie House classification of diabetic retinopathy [22]. All study participants were recruited and examined at the Department of Ophthalmology, Sveti Duh Clinical Hospital in Zagreb. Non-inclusion criteria encompassed subjects previously treated with intravitreal steroids or anti-VEGF therapy, subjects who had previously undergone vitreoretinal surgery, and subjects with other retinal diseases (senile macular degeneration, central retinal artery occlusion and/or veins and branches), subjects on systemic corticosteroid therapy or cytostatics, all subjects with poorly controlled cardiovascular status, pregnant women, and women unable to exclude the possibility of pregnancy with certainty.

The study was approved by the Ethics Committee of the Sveti Duh Clinical Hospital, and was conducted at the Department of Ophthalmology of the hospital from June 2012 to February 2013. All applicable guidelines were followed: Basics of Good Clinical Practice, Helsinki Declaration,

Croatian Healthcare Act, and Patient Rights Act. All patients gave their informed consent to participate in the study, after receiving detailed information from their ophthalmologist.

*Surgical procedure:* Vitreous samples (1.5–2.0 ml) were obtained by the standard vitreoretinal aspiration procedure, pars plana vitrectomy, and all procedures were conducted by the same vitreoretinal surgeon. Vitreal samples were taken immediately after setting the trocars (before any other surgical manipulation) and before turning on the infusion system to avoid dilution (filtered air was insufflated to retain volume). Samples were centrifuged within one hour after surgery for 10 min at 15000 ×g at 4 °C. After centrifugation, the liquid portion (vitreous) was separated and stored at –80 °C until analysis. Blood samples for serum analysis (5 ml) were simultaneously collected from the cubital vein, in vials containing EDTA, and were centrifuged at 1200 ×g at 4 °C. Serum samples were stored at –80 °C. At the time of analysis, all samples were dissolved at room temperature and, if necessary, centrifuged again. All analyses, sample loading, and reagent mixture additions were performed in an array manner using equal sample loading by multichannel pipettes.

*Chemicals:* Chemicals used in the biochemical analysis were purchased from Sigma Chemical Company. Bovine heart cytochrome C (Type VI) and human blood SOD (Type I, lyophilized powder, 2400 U/mg protein), BSA, xantine, xantine oxidase, 2-thiobarbituric acid, dodecyl sulfate sodium salt, and 1,1,3,3-tetrametoxyp propane were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade: OxiSelect™ AOPP Assay Kit, Lipid Hydroperoxide Assay kit (Cayman Chemical Company, Ann Arbor, MI), VEGF kit (Enzo Life Sciences, Farmingdale, NY), and glucose assay kit (GOD-PAP method, HUMAN™).

*Glucose assay:* Glucose levels in serum were measured by a glucose liquicolor (GOD-PAP method) HUMAN™ enzymatic colorimetric test for glucose detection following the manufacturer's instructions. Briefly, 1000 µl of reagent mixture (glucose oxidase, 4-aminoantipyrinephenol, peroxidase, and mutarotase) was added to 10 µl of the sample. Absorbance was measured at 500 nm with a Libro S22 spectrophotometer (Biochrom, UK). Calculation was made by dividing absorbance of the sample by absorbance of a supplied glucose standard solution and multiplying by factors of 100 and 5.55. The glucose concentration was expressed as mmol/l.

*VEGF assay:* Vascular endothelial growth factor (VEGF) was measured using a VEGF ELISA kit (human; Enzo Life Sciences, USA). All kit reagents were prepared according to the manufacturer's instructions. A polyclonal antibody against human VEGF-16VEGF labeled with the enzyme

horseradish peroxidase was added to samples. The measured optical density was read at 450 nm and was directly proportional to the concentration of human VEGF in the standards or samples. The concentration of VEGF was calculated from a calibration curve and expressed as pg/l.

*Advanced oxidation protein products (AOPP) assay:* AOPP was assayed with the OxiSelect™ AOPP Assay Kit according to the manufacturer's instructions. AOPP content was determined by comparing the test sample with the chloramine standard curve. Briefly, 200 µl samples or standards were added to separate wells of the microtiter plate (Biorad, Hercules, CA). A total of 10 µl chloramine reaction initiator was added. The absorbance of each well was recorded immediately on a spectrophotometric plate reader using a wavelength of 340 nm. Results were calculated according to a standard curve and expressed as µM.

*Lipid hydroperoxide (LPO) assay:* Lipid hydroperoxide was assayed with the Lipid Hydroperoxide Assay kit (Cayman Chemical Company) by direct measurement of redox reaction with iron ions according to the kit manual. Briefly, the solution LPO Assay Extract R was added to test samples. To this mixture, 1 ml cold chloroform solution was added and blended. A total of 450 µl of the chloroform-methanol mixture was added to 500 µl of extract chloroform sample, followed by a 50 µl mixture of chromogen, which turned purple. Absorbance was measured at 500 nm with a Spectro UVD-3500 spectrophotometer (Labomed Inc., Los Angeles, CA). From the calibration curve of LPO, we calculated the concentration of LPO in each sample according to the formula specified by the manufacturer and expressed as µM.

*Malondialdehyde (MDA) assay:* The presence of lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA). A total of 200 µl supernatant was mixed with 200 µl 8.1% aqueous sodium dodecyl sulfate, 1.5 ml 20% aqueous acetic acid (pH 3.5), and 1.5 ml 0.81% aqueous thiobarbituric acid and heated for 60 min at 95 °C. After cooling samples on ice, absorbance was measured at 532 nm and 600 nm with a Libro S22 spectrophotometer (Biochrom, Cambridge, UK). The total absorbance was determined using the formula  $A_{\text{total}} = A_{532} - A_{600}$ . An array of known concentrations of tetramethoxypropane was used for creating the calibration curve using the same protocol as for the homogenized samples. MDA values are presented as nmol/mL.

*Glutathione assay (GSH) assay:* The glutathione assay is a modification of the method first described by Tietze [23]. Briefly, in a 96-well plate, 40 µl 10 mM 5–5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent) was added to 20 µl sample supernatant pre-treated with 40 µl 0.035M

HCL. This mixture was incubated for 10 min. DTNB reacts with GSH to form chromospheres. The absorbance of these chromogens was measured at 412 nm in an ELISA plate reader (BIORAD). Then, 100 µl reaction mixture (9980 µl 0.8 mM NADPH and 20 µl glutathione reductase, 0.2 U/ml) was added and the absorbance was read at 412 nm every minute for 5 min. The results were calculated from the standard curve of array of dilutions of glutathione (GSH). Concentrations are presented as µmol/ml.

*Total superoxide dismutase (SOD) assay:* The measure of SOD activity is calculated from the percentage of inhibition of the reaction of xantine oxidation by xantine oxidase (optimized reaction ratio  $\Delta A / \text{min} \approx 0.025$ ), which creates a superoxide anion as a substrate for SOD. The superoxide anion not used by the enzyme SOD oxidizes the cytochrome. For determination of SOD activity, 25 µl of undiluted sample were mixed with 1.45 ml of the reaction mix (cytochrome C, 0.05 mM; xantine, 1 mM mixed to a 10:1 ratio with addition of DTNB). To this mixture, 20 µl xantine oxidase 0.4 Uml<sup>-1</sup> was added to start a reaction. The reaction was measured over 3 min at 550 nm. The absorbance and percentage of inhibition were compared to the calibration curve created with different dilutions of SOD. Enzyme values are presented as U/ml.

*Statistical analysis:* Statistics were based on non-parametric methods, due to the small sample size and non-normal data distribution (verified by the Kolmogorov–Smirnov test). The Mann–Whitney U test was used to analyze numerical variables between groups. Spearman's rank correlation was used for correlation analysis within and between measured parameters in the vitreous and serum of both groups of patients (NDED and PDR). Analyses were performed using SPSS version 17 (SPSS Inc., Chicago, IL). The level of statistical significance was set at  $p \leq 0.05$ .

## RESULTS

A comparison of gender-dependent differences (Table 1) revealed that there were no statistically significant gender differences in the measured parameters in the vitreous or serum, except a single detected difference in the NDED group, where serum MDA was significantly lower ( $p \leq 0.05$ ) in male than in female patients. However, within the same gender groups, there were significant differences ( $p \leq 0.05$ ) between the NDED and PDR groups in the majority of measured parameters (Table 1).

Trends of statistical differences in the measured parameters between the NDED and PDR groups by age distribution (Table 2) were similar to trends within the sexes (Table 1). The correlation of the measured parameters and age of patients included in the study (Table 2) revealed a significant negative



correlation ( $p \leq 0.05$ ) of serum MDA and SOD activity with increased age in PDR patients, and serum VEGF was positively correlated with increasing age.

The analysis of serum glucose of NDED patients and PDR patients at the time of the procedure (Figure 1) revealed that glucose levels were as expected, i.e., were significantly higher ( $p \leq 0.05$ ) in PDR patients than in NDED patients. However, serum glucose values did not correlate significantly with changes of the measured parameters in either group (Table 3), with the exception of a slight significant positive correlation with MDA in the NDED group.

Vitreous VEGF levels were significantly increased ( $p \leq 0.05$ ), almost 10-fold, in the PDR group compared to NDED patients (Figure 2). Serum VEGF of the PDR group was also increased significantly ( $p \leq 0.05$ ; Figure 2). However, since the scale on Figure 2 is set for vitreal VEGF, the serum VEGF range is provided here (serum VEGF: NDED range 2.0–104 pg/ml; PDR range 5.0–760 pg/ml).

The analysis of advanced oxidized protein product (AOPP) levels (Figure 3) revealed no statistical differences between NDED and PDR patients. Serum AOPP levels in NDED patients were only slightly significantly ( $p \leq 0.05$ ) higher than in the vitreous. However, serum AOPP levels were significantly higher ( $p \leq 0.05$ ) in PDR than in NDED patients.

Lipid peroxidation (LPO; Figure 4) was almost equal in the vitreous and serum in the NDED group. In the PDR group, LPO values were slightly but significantly higher ( $p \leq 0.05$ ) in serum than in the vitreous. In a comparison between groups, LPO values were significantly ( $p \leq 0.05$ ) higher in the PDR group, with an almost five-fold increase of the peroxydized lipid concentration. The LPO analysis marked the most prominent change of all the analyzed oxidative stress markers.

Similarly, MDA (Figure 5) is also a lipid peroxidation marker. It was established that serum levels were higher ( $p \leq 0.05$ ) than vitreous values in the PDR group. In the NDED group, serum and vitreous MDA values showed no significant difference. However, vitreous and serum MDA values were significantly higher ( $p \leq 0.05$ ) in the PDR than in the NDED group.

The activity of total superoxide dismutase (SOD; Figure 6) was slightly but significantly ( $p \leq 0.05$ ) lower in serum than in the vitreous of the NDED subjects. In PDR patients, the vitreal SOD activity was slightly but significantly ( $p \leq 0.05$ ) lower than in NDED patients, while serum SOD activity was significantly higher ( $p \leq 0.05$ ) compared to NDED patients.

Glutathione levels (GSH) were significantly ( $p \leq 0.05$ ) higher in the serum than in the vitreous of both NDED and PDR groups; however, no significant differences were detected in the serum or vitreous GSH levels between the groups (Figure 7).

**TABLE 1. MEASURED PARAMETERS AND STATISTICAL DIFFERENCES WITHIN AND BETWEEN GENDERS IN NON-DIABETIC PATIENTS WITH EYE DISORDERS (NDED) AND PROLIFERATIVE DIABETIC RETINOPATHY (PDR) PATIENTS.**

Parameter	Group	Vitreous		Serum	
		Male	Female	Male	Female
		Mean $\pm$ S.E	Mean $\pm$ S.E	Mean $\pm$ S.E	Mean $\pm$ S.E
VEGF (pg/l)	NDED	176.3 $\pm$ 34.8	213.5 $\pm$ 50.5	18.9 $\pm$ 2.7	29.0 $\pm$ 6.2
	PDR	2048.9 $\pm$ 408.4*	1127.2 $\pm$ 23.6*	70.7 $\pm$ 26.7*	50.0 $\pm$ 16.8
AOPP( $\mu$ M)	NDED	44.0 $\pm$ 12.3	43.9 $\pm$ 25.6	231.9 $\pm$ 19.6	250.3 $\pm$ 18.8
	PDR	68.6 $\pm$ 19.0	51.4 $\pm$ 20.6	379.4 $\pm$ 46.8*	522.1 $\pm$ 134.1*
LPO ( $\mu$ M)	NDED	27.0 $\pm$ 2.4	33.0 $\pm$ 4.9	26.2 $\pm$ 2.3	26.2 $\pm$ 1.5
	PDR	145.8 $\pm$ 6.3*	135.6 $\pm$ 10.9*	168.1 $\pm$ 6.9*	159.0 $\pm$ 8.7*
MDA (nmol/ml)	NDED	55.8 $\pm$ 10.9	58.1 $\pm$ 12.5	31.9 $\pm$ 3.1	40.4 $\pm$ 6.4#
	PDR	101.3 $\pm$ 7.6*	87.6 $\pm$ 18.4*	151.5 $\pm$ 12.5*	156.2 $\pm$ 25.7*
SOD (U/ml)	NDED	76.3 $\pm$ 11.9	50.5 $\pm$ 7.2	24.3 $\pm$ 1.3	26.7 $\pm$ 2.0
	PDR	30.5 $\pm$ 2.5*	28.5 $\pm$ 3.8*	158.8 $\pm$ 23.5*	122.1 $\pm$ 13.7*
GSH ( $\mu$ mol/ml)	NDED	41.1 $\pm$ 1.1	38.9 $\pm$ 0.4	90.5 $\pm$ 6.32	80.2 $\pm$ 5.4
	PDR	43.7 $\pm$ 1.8	48.8 $\pm$ 5.8	73.5 $\pm$ 2.9*	69.9 $\pm$ 5.7*

\* values are statistically different ( $p \leq 0.05$ ) from the same parameter of non-diabetic patients with eye disorders (NDED) within the same gender group (column), i.e., males in NDED versus PDR; females in NDED versus PDR. # values are statistically different between genders within non-diabetic patients with eye disorders (NDED) and with proliferative diabetic retinopathy (PDR) patients ( $p \leq 0.05$ ).

**TABLE 2. MEAN VALUES OF MEASURED PARAMETERS BY AGE GROUPS AND THE CORRELATION ANALYSIS OF MEASURED PARAMETERS WITH AGE IN NON-DIABETIC PATIENTS WITH EYE DISORDERS (NDED) AND PROLIFERATIVE DIABETIC RETINOPATHY (PDR) PATIENTS.**

Parameter	Group	Mean by age groups (years) ± S.E					Spearman correlation (ρ)
		≤60	61-65	66-70	71-75	≥76	
VEGF (pg/l)	NDED	86.8 ± 10.8	217.1 ± 99.3	224.3 ± 104.5	238.0 ± 56.1	139.5 ± 41.5	0.224
	PDR	1594.3 ± 473.7*	2274.1 ± 804.0*	2268.0 ± 843.9*	1401.6 ± 222.7*	1158.7 ± 514.4*	-0.032
AOPP (μM)	NDED	29.0 ± 16.5	62.6 ± 54.6	56.2 ± 46.6	39.1 ± 12.6	44.0 ± 13.9	-0.242
	PDR	66.5 ± 22.3	33.4 ± 10.6	53.2 ± 32.6	81.0 ± 46.5	143.0 ± 104.3	0.022
LPO (μM)	NDED	26.0 ± 3.5	33.8 ± 6.1	34.8 ± 4.9	25.0 ± 4.1	27.4 ± 4.8	-0.195
	PDR	153.3 ± 9.7*	139.2 ± 7.5*	117.0 ± 14.1*	151.0 ± 18.9*	147.0 ± 18.8*	-0.178
MDA (nmol/ml)	NDED	33.0 ± 10.1	60.9 ± 22.2	99.8 ± 43.1	46.5 ± 12.3	61.3 ± 14.5	0.072
	PDR	91.7 ± 5.5*	103.5 ± 4.7	48.4 ± 11*	57.5 ± 42.6	123.2 ± 39.5*	-0.011
SOD (U/ml)	NDED	48.3 ± 3.4	47.5 ± 18.9	90.3 ± 32.1	72.2 ± 18.1	72.5 ± 14.7	0.307
	PDR	25.6 ± 3.5*	37.3 ± 3.7	24.8 ± 5.8*	29.0 ± 8.3*	30.0 ± 2.1*	0.100
GSH (μmol/ml)	NDED	36.6 ± 7.6	44.2 ± 5.0	41.5 ± 2.5	38.5 ± 0.3	39.1 ± 0.3	-0.098
	PDR	43.6 ± 3.1	43.3 ± 2.7	50.7 ± 7.9	52.0 ± 11.8	41.2 ± 0.8	0.070
<b>Serum</b>							
VEGF (pg/l)	NDED	≤60	61-65	66-70	71-75	≥76	Spearman correlation (ρ)
	PDR	15.3 ± 4.3	27.7 ± 8.7	21.0 ± 6.3	17.2 ± 3.9	25.8 ± 6.6	-0.136
AOPP (μM)	NDED	34.2 ± 6.6*	61.3 ± 20.2	175.1 ± 146.3	36.6 ± 4.4*	72.4 ± 21.8*	0.407#
	PDR	238.0 ± 42.6	283.0 ± 35.3	198.1 ± 44.2	241.1 ± 25.0	240.8 ± 24.9	-0.176
LPO (μM)	NDED	451.2 ± 77.1*	501.4 ± 109.8*	438.2 ± 108.0*	235.6 ± 79.7	275.0 ± 94.0	-0.236
	PDR	21.0 ± 3.8	23.4 ± 2.6	19.0 ± 3.7	26.8 ± 2.5	30.4 ± 3.5	0.289
MDA (nmol/ml)	NDED	177.6 ± 7.6*	162.4 ± 7.3*	147.0 ± 24.2*	176.0 ± 15.7*	150.2 ± 25.6*	-0.260
	PDR	27.4 ± 4.4	32.9 ± 6.7	29.5 ± 8.4	30.1 ± 7.4	31.6 ± 6.4	-0.156
SOD (U/ml)	NDED	171.1 ± 12.1*	165.9 ± 33.5*	132.2 ± 11.9*	128.6 ± 16.0*	107.4 ± 3*	-0.427#
	PDR	28.4 ± 4.0	22.2 ± 2.1	22.0 ± 2.9	23.6 ± 2.1	27.1 ± 2.0	0.179
GSH (μmol/ml)	NDED	208.5 ± 43.6*	122.1 ± 14.01*	95.3 ± 11.9*	107.2 ± 45.6*	126.8 ± 12.2*	-0.443#
	PDR	73.0 ± 8.0	101.9 ± 21.7	92.8 ± 13.0	89.7 ± 8.4	80.5 ± 6.8	-0.111
		70.9 ± 4.8	74.6 ± 4.3	85.1 ± 1.7	60.0 ± 14.9	70.7 ± 3.9	-0.062

\* values are statistically different (p≤0.05) from the same parameter of non-diabetic patients with eye disorders (NDED) within the same age group (column). # values are statistically significantly correlated (p≤0.05) with age within the non-diabetic patients with eye disorders (NDED) or proliferative diabetic retinopathy (PDR) patients. ρ - Spearman correlation analysis coefficient; p- level of statistical significance (p≤0.05) of Spearman correlation analysis.

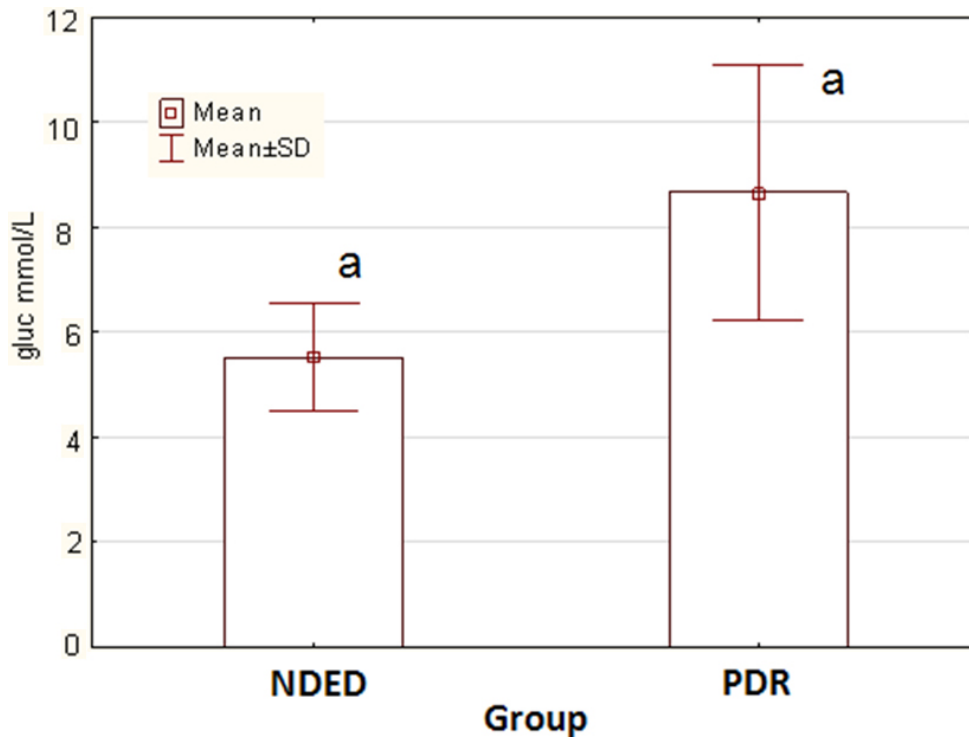


Figure 1. Average serum glucose concentrations in non-diabetic patients with eye disorders (NDED) and proliferative diabetic retinopathy (PDR) patients. NDED group (n=50); PDR group (n=37). <sup>a</sup>Columns are significantly different (p<0.05).

The Spearman correlation coefficients of the measured parameters in the NDED subjects are presented in Table 4 and for PDR patients in Table 5. In the NDED group, the only significant correlation between the measured parameters was the negative correlation between GSH and SOD in serum (Table 4,  $\rho = -0.627$ ;  $p < 0.001$ ). In PDR, however, several correlations occurred as a consequence of the pathological condition and new allostasis (Table 5). The significantly ( $p \leq 0.05$ ) strongest ones ( $\rho \leq \pm 0.600$ ) were between vitreous LPO and serum SOD (Table 5, Figure 8A), serum SOD and LPO (Table 5, Figure 8B), vitreous LPO and serum LPO (Table 5, Figure 8C), and vitreous VEGF and serum SOD (Table 5, Figure 8D).

## DISCUSSION

Investigating the dynamics and correlative ties of oxidative stress markers in homeostatically linked physiologic fluids, i.e., the vitreous and serum of patients having proliferative diabetic retinopathy (PDR), might reveal possible markers and their ratios for disease control and monitoring progression.

This study of the links and correlations between the measured parameters indicated that all parameters assayed in the serum or eye of NDED subjects had  $\rho$  (Spearman correlation coefficient) values near zero. Conclusively, markers in the vitreous or serum were physiologically independent in the NDED group (Table 4). Conversely, in PDR patients, diabetic

retinopathy and increased oxidative stress resulted in several statistically significant correlations between the measured parameters in the vitreous and serum (Table 5). This is likely due to the fact that the blood-retinal barrier becomes more permeable in diabetes, as has been shown experimentally [8].

Age (Table 2) was found to be a partially contributing factor, i.e., with increasing age of diabetic patients, there was a significant increase of serum VEGF and a reduction of serum SOD and MDA, but age did not influence the vitreal changes. One must remember that the overall serum SOD activity and MDA levels were 3–4 times higher in PDR than in NDED patients, and the correlation with age occurs in such a higher range of pathophysiological allostasis (Figure 5 and Figure 6).

The analysis of glucose levels (Figure 1) shows that, at the time of analysis in diabetic patients (fasting patients on the day of operation), it may be statistically but is not prominently higher. Parameters were not significantly correlated to glucose levels at the time of measurement (Table 3). Rather, it is the long period of fluctuations of all biochemical parameters over time that results in the imbalance of the antioxidant defense system.

VEGF was found to be increased in the vitreous of PDR patients (Figure 2), similar to [24-26]. However, in this study, a moderate yet significant positive correlation ( $\rho = 0.357$

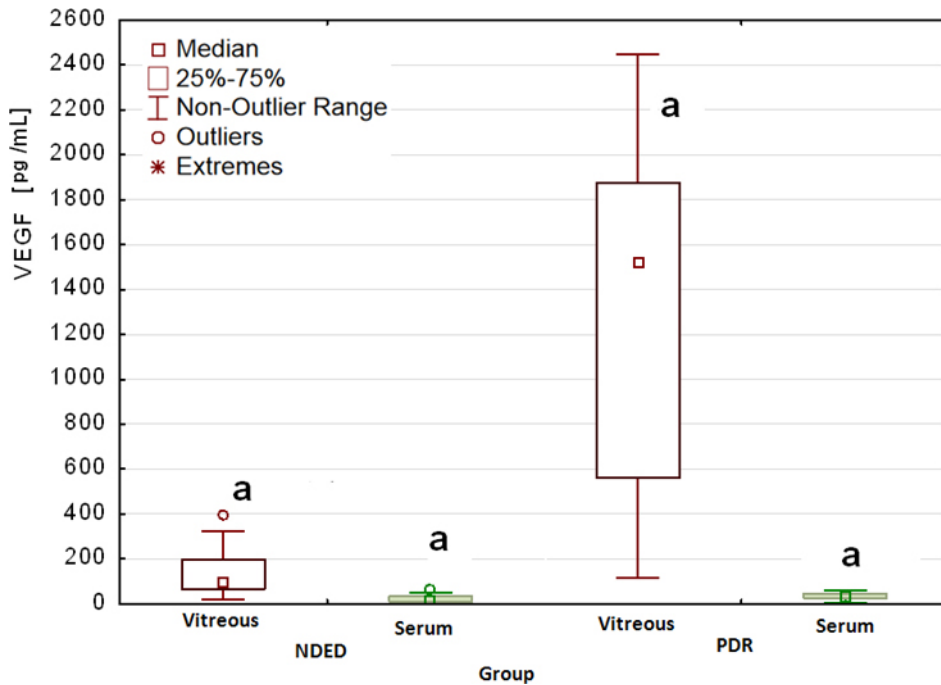


Figure 2. Relationships of vitreous and serum VEGF values in non-diabetic patients with eye disorders (NDED) and proliferative diabetic retinopathy (PDR) patients. NDED group (n=50); PDR group (n=37). <sup>a</sup>Boxes are significantly different (p<0.05).

TABLE 3. CORRELATION OF MEASURED PARAMETERS WITH SERUM GLUCOSE IN NON-DIABETIC PATIENTS WITH EYE DISORDERS (NDED) AND PROLIFERATIVE DIABETIC RETINOPATHY (PDR) PATIENTS.

Parameter	Group	Serum glucose			
		Vitreous		Serum	
		Spearman coefficient of correlation (ρ)	p	Spearman coefficient of correlation (ρ)	p
AGE (years)	NDED	/	/	0.078	0.695
	PDR	/	/	-0.217	0.257
VEGF (pg/l)	NDED	-0.006	0.968	-0.143	0.357
	PDR	0.249	0.155	-0.133	0.452
AOPP (μM)	NDED	-0.172	0.381	0.225	0.240
	PDR	-0.098	0.580	0.018	0.917
LPO (μM)	NDED	0.100	0.512	0.257	0.088
	PDR	-0.076	0.666	-0.099	0.5767
MDA (nmol/ml)	NDED	0.132	0.517	0.302 <sup>#</sup>	0.045
	PDR	0.149	0.529	0.207	0.262
SOD (U/ml)	NDED	0.184	0.253	-0.046	0.774
	PDR	-0.101	0.592	0.071	0.729
GSH (μmol/ml)	NDED	-0.246	0.111	-0.115	0.467
	PDR	0.133	0.466	0.315	0.116

<sup>#</sup>values are statistically significantly correlated (p<0.05) with glucose within the non-diabetic patients with eye disorders (NDED) or proliferative diabetic retinopathy (PDR) group. ρ - Spearman correlation analysis coefficient; p- level of statistical significance (p<0.05) of Spearman correlation analysis.



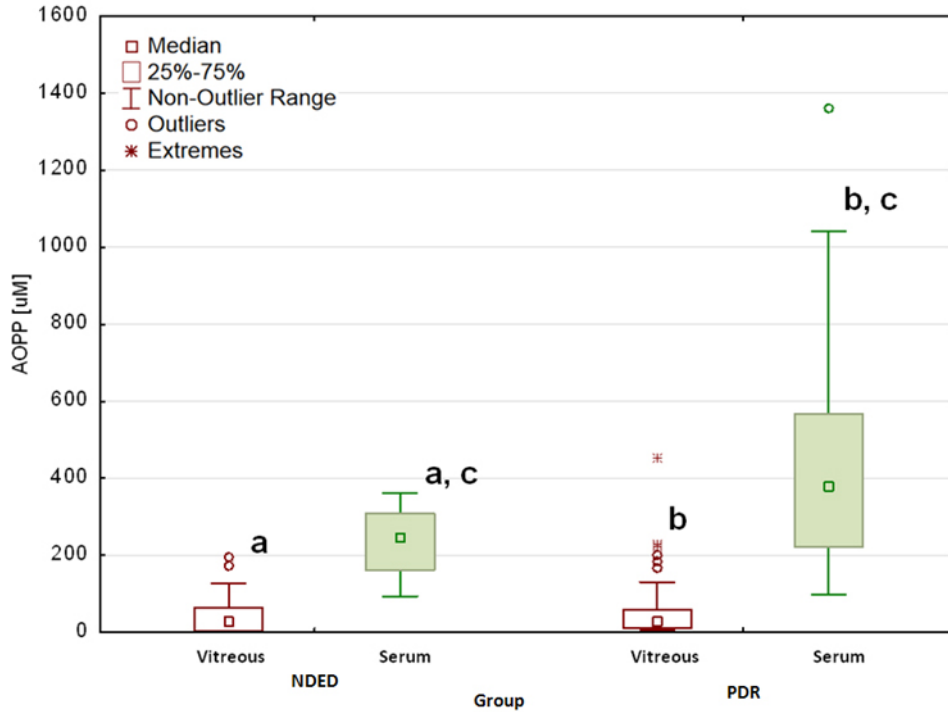


Figure 3. Relationships of vitreous and serum AOPP values in non-diabetic patients with eye disorders (NDED) and proliferative diabetic retinopathy (PDR) patients. NDED group (n=50); PDR group (n=37). <sup>a,b,c</sup>Boxes bearing the same superscript letter are significantly different ( $p \leq 0.05$ ).

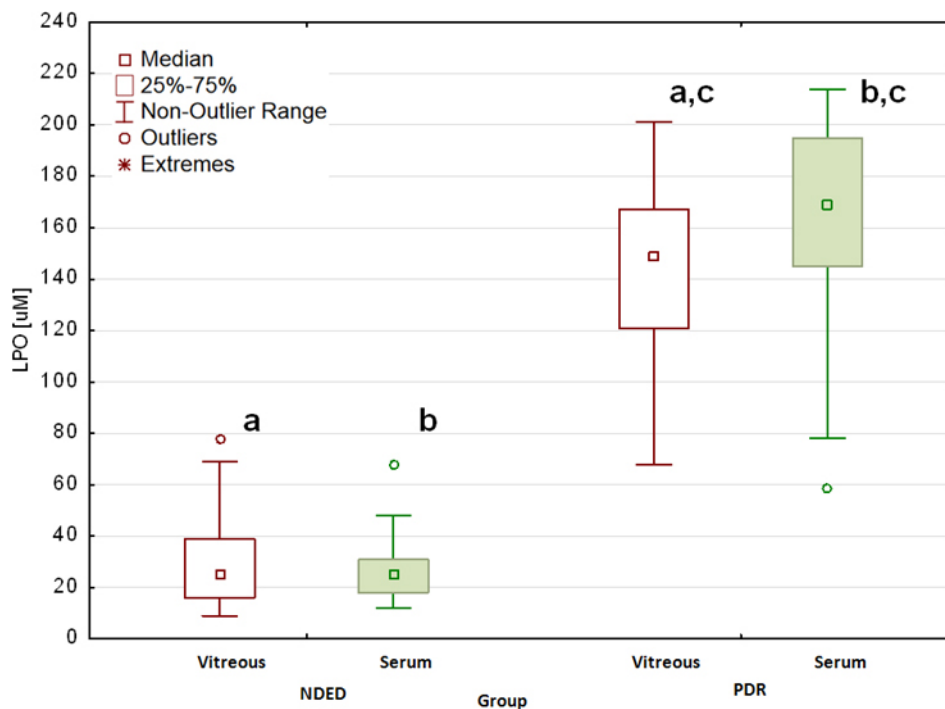


Figure 4. Relationships of vitreous and serum LPO values in non-diabetic patients with eye disorders (NDED) and proliferative diabetic retinopathy (PDR) patients. NDED group (n=50); PDR group (n=37). <sup>a,b,c</sup>Boxes bearing the same superscript letter are significantly different ( $p \leq 0.05$ ).

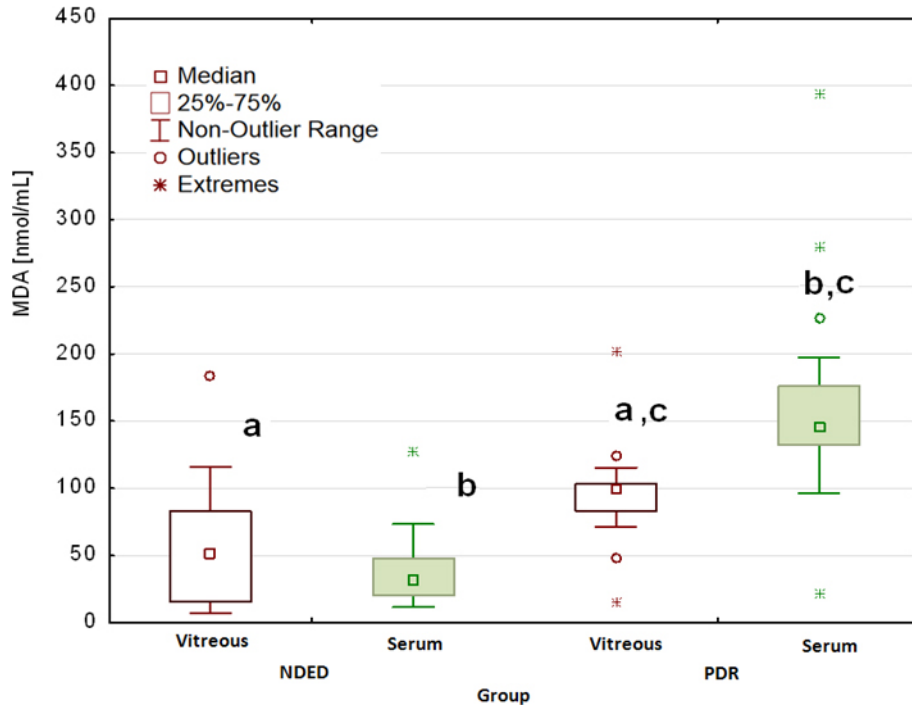


Figure 5. Relationships of vitreous and serum MDA values in non-diabetic patients with eye disorders (NDED) and proliferative diabetic retinopathy (PDR) patients. NDED group (n=50); PDR group (n=37). <sup>a,b,c</sup>Boxes bearing the same superscript letter are significantly different ( $p \leq 0.05$ ).

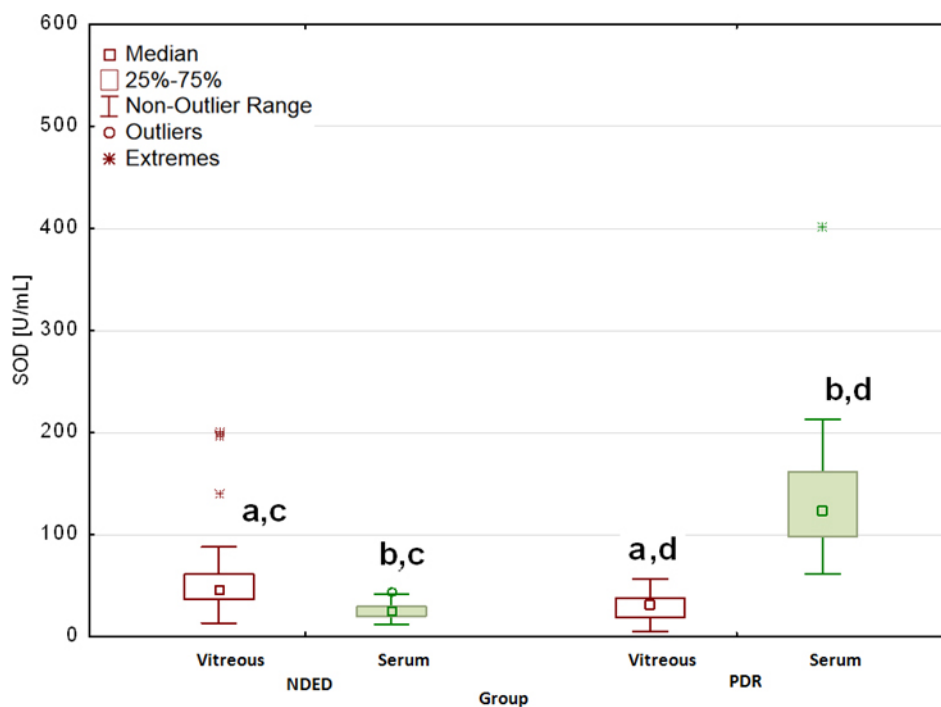


Figure 6. Relationships of vitreous and serum SOD activity levels in non-diabetic patients with eye disorders (NDED) and proliferative diabetic retinopathy (PDR) patients. NDED group (n=50); PDR group (n=37). <sup>a,b,c,d</sup>Boxes bearing the same superscript letter are significantly different ( $p \leq 0.05$ ).

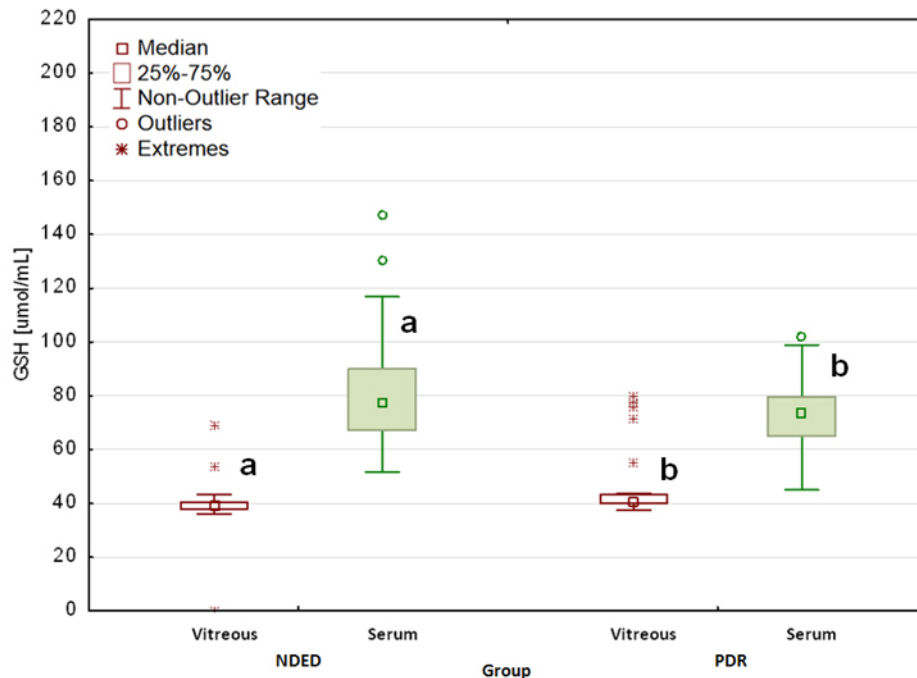


Figure 7. Relationships of vitreous and serum GSH values in non-diabetic patients with eye disorders (NDED) and proliferative diabetic retinopathy (PDR) patients. NDED group (n=50); PDR group (n=37). <sup>a,b,c,d</sup>Boxes bearing the same superscript letter are significantly different ( $p \leq 0.05$ ).

and  $p=0.001$ ; Table 5) was found between the vitreous and serum VEGF in the PDR group. We note that, in diabetes, microvascularization in other tissues also contribute to an increase in serum VEGF, though this significant correlation between serum and vitreous levels may suggest an important physiologic link between these humoral compartments [8]. As such, serum levels could perhaps reflect intraocular changes.

AOPPs are structurally similar to advanced glycation end-product (AGE) and exert similar biologic activity. Serum AOPP levels are elevated in patients with renal complications, atherosclerosis, and diabetes [27]. The present study revealed no statistically significant differences in vitreous AOPP between the NDED and PDR patients (Figure 1). The established difference between higher serum than vitreal values of AOPP can be explained by the higher protein content in serum than in the vitreous, and higher values of serum oxidized proteins in PDR than in NDED patients. Data on AOPP levels in the vitreous of diabetic patients with PDR disease is scarce, though serum AOPP increases in diabetes have been reported, together with an increase in oxidized albumin and protein carbonyls [27-31]. The correlation analysis showed that AOPP increased independently from other parameters, since it was not significantly correlated to changes in other parameters in either the vitreous or serum. Accordingly, the AOPP method used here does not reflect vitreous oxidative stress and is more specific for plasmatic oxidative stress products. Glutathione (GSH), a small peptide, showed no

prominent changes. There are reports of unchanged thiol levels in PDR patients [27]. Others, however, have reported total thiol levels and GSH depletion in serum and vitreous in human subjects with PDR [32], though most of the reports are on animal models [33-35].

The results and comparison of lipid peroxidation markers using direct measures of lipid peroxidation (LPO) and indirect measures of the lipid peroxidation byproduct malondialdehyde (MDA) indicated that the LPO measurement method (Figure 4) showed a more prominent difference (5-7 times higher increase in serum and vitreous) than the MDA method (2-3 times increase in serum and vitreous) in the PDR group (Figure 5). There are no literature reports assessing lipid peroxidation (LPO) using the method described here, though earlier studies measuring lipid peroxidation using other markers yielded significantly increased results [35]. The present results on MDA changes in the vitreous of diabetic patients with PDR are in accordance with the studies by Mandal et al. [36] and Manciano et al. [37], which showed an increase in vitreal MDA in PDR patients. They also indicated that lipid peroxidation in diabetic patients with PDR is a highly pronounced process in the humoral parts of the organism, and lipid peroxidation appears to be highly responsible for induced oxidative stress in diabetic patients [38,39]. The LPO method is more sensitive compared to methods that measure lipid peroxidation byproducts, such as MDA. LPO offers a better picture of the extent of lipid peroxidation than

**TABLE 4. CORRELATION ANALYSIS OF MEASURED PARAMETERS IN VITREOUS AND SERUM OF NON-DIABETIC PATIENTS WITH EYE DISORDERS (NDED) PATIENTS.**

Parameter	Vitreous						Serum					
	VEGF (pg/l)	AOPP (µM)	LPO (µM)	MDA (nmol/ml)	SOD (U/ml)	GSH (µmol/ml)	VEGF (pg/l)	AOPP (µM)	LPO (µM)	MDA (nmol/ml)	SOD (U/ml)	GSH (µmol/ml)
<b>Vitreous</b>												
VEGF (pg/l)	/	-0.047	0.161	0.007	-0.121	-0.151	0.234	-0.017	0.097	-0.119	-0.027	0.022
AOPP (µM)	0.807	/	0.053	-0.141	0.075	0.058	-0.221	0.048	0.114	-0.122	-0.052	-0.092
LPO (µM)	0.269	0.781	/	0.176	0.045	0.017	-0.073	-0.199	0.215	-0.046	0.168	-0.201
MDA (nmol/ml)	0.971	0.487	0.369	/	-0.032	-0.131	-0.326	0.296	-0.029	-0.257	0.105	0.012
SOD (U/ml)	0.433	0.704	0.767	0.876	/	-0.058	0.021	-0.096	0.137	0.126	0.104	-0.239
GSH (µmol/ml)	0.309	0.763	0.909	0.508	0.702	/	-0.048	0.089	-0.001	0.021	0.336	-0.244
<b>Serum</b>												
VEGF (pg/l)	0.105	0.242	0.621	0.091	0.892	0.748	/	0.023	0.111	-0.124	-0.072	0.077
AOPP (µM)	0.927	0.801	0.284	0.126	0.622	0.635	0.903	/	0.127	-0.457	0.222	0.025
LPO (µM)	0.508	0.551	0.131	0.884	0.365	0.994	0.446	0.497	/	0.031	0.301	-0.119
MDA (nmol/ml)	0.431	0.532	0.756	0.187	0.411	0.893	0.412	0.011	0.841	/	0.093	-0.239
SOD (U/ml)	0.862	0.794	0.265	0.611	0.513	0.024	0.641	0.247	0.042	0.543	/	-0.627#
GSH (µmol/ml)	0.882	0.635	0.172	0.951	0.113	0.099	0.611	0.897	0.421	0.102	<0.001	/

# values are significantly correlated (p≤0.05) Values above the diagonal empty cells (/) represent the Spearman correlation coefficient (ρ); values below the diagonal empty cells represent the level of statistical significance (p≤0.05) of the correlation.

**TABLE 5. CORRELATION ANALYSIS OF MEASURED PARAMETERS IN VITREOUS AND SERUM OF PROLIFERATIVE DIABETIC RETINOPATHY (PDR) PATIENTS.**

Parameter	Vitreous						Serum					
	VEGF (pg/l)	AOPP (µM)	LPO (µM)	MDA (nmol/ml)	SOD (U/ml)	GSH (µmol/ml)	VEGF (pg/l)	AOPP (µM)	LPO (µM)	MDA (nmol/ml)	SOD (U/ml)	GSH (µmol/ml)
<b>Vitreous</b>												
VEGF (pg/l)	/	0.129	-0.081	0.309	-0.477 <sup>#</sup>	0.198	0.357 <sup>#</sup>	0.045	-0.008	0.632 <sup>#</sup>	-0.102	
AOPP (µM)	0.298	/	0.159	-0.048	0.021	0.079	-0.049	0.147	-0.042	0.123	-0.061	
LPO (µM)	0.471	0.198	/	0.457 <sup>#</sup>	-0.441 <sup>#</sup>	0.387 <sup>#</sup>	0.367 <sup>#</sup>	0.838 <sup>#</sup>	0.095	0.742 <sup>#</sup>	-0.277	
MDA (nmol/ml)	0.031	0.744	0.001	/	-0.251	-0.021	0.135	0.346 <sup>#</sup>	-0.184	0.406 <sup>#</sup>	0.036	
SOD (U/ml)	<0.001	0.872	<0.001	0.086	/	-0.238	-0.141	-0.421 <sup>#</sup>	0.075	-0.312 <sup>#</sup>	-0.049	
GSH (µmol/ml)	0.072	0.533	<0.001	0.886	0.035	/	0.239	0.371 <sup>#</sup>	0.016	0.361 <sup>#</sup>	-0.259	
<b>Serum</b>												
VEGF (pg/l)	0.001	0.697	0.001	0.355	0.223	0.029	/	0.402 <sup>#</sup>	-0.049	0.354 <sup>#</sup>	-0.031	
AOPP (µM)	0.059	0.544	0.013	0.169	0.264	0.137	0.216	0.375 <sup>#</sup>	0.012	0.350 <sup>#</sup>	0.116	
LPO (µM)	0.356	0.235	<0.001	0.015	<0.001	<0.001	<0.001	/	0.141	0.778 <sup>#</sup>	-0.214	
MDA (nmol/ml)	0.941	0.743	0.395	0.215	0.521	0.887	0.665	0.208	/	0.119	-0.220 <sup>#</sup>	
SOD (U/ml)	<0.001	0.371	<0.001	0.006	0.011	0.002	0.002	<0.001	0.318	/	-0.418 <sup>#</sup>	
GSH (µmol/ml)	0.391	0.663	0.016	0.811	0.692	0.028	0.798	0.066	0.050	<0.001	/	

# values are significantly correlated (p≤0.05) Values above the diagonal empty cells (/) represent the Spearman correlation coefficient (ρ); values below the diagonal empty cells represent the level of statistical significance (p≤0.05) of the correlation.



MDA. This was confirmed by the significant correlations between LPO in the vitreous and serum. MDA was the only serum variable in the study group to remain independent from the other parameters. Its  $\rho$  value remained similar as in the NDED group. This indicates that the MDA change in serum does not reflect changes in the vitreous but rather depends on systemic sources from other tissues. In addition, the determined  $\rho$  value for LPO showed a significant positive association with increased LPO concentrations in the eye and in serum, but also with vitreous MDA and SOD and serum VEGF, AOPP, SOD, and especially with serum LPO. These results indicate that the increase in overall serum LPO levels, compared to normal serum levels, is approximately twice the increase in lipid peroxidation in the eye. Given that previous studies [35,39] have shown a significant correlation between the increase of LPO in the vitreous and an increase in the expression of vascular endothelial growth factors (VEGF), it appears that LPO determination in serum might be a good predictor for the onset of oxidative stress within the vitreous. Furthermore, Table 5 and Figure 8 show that a pronounced

correlation change was found for serum LPO and serum SOD activity. A relatively strong and physiologically significant correlation was recorded between serum SOD activity and vitreous LPO levels. Unlike the high serum SOD activity, the ocular activity of SOD shown in Figure 6 is likely to decrease slightly compared to NDED patients. Yildirim et al. [17] reported no changes in serum SOD activity in PDR patients. This can be explained by the saturation of the ocular enzyme by its substrate (superoxide radical), which is a free radical whose concentration is elevated during oxidative stress. On the other hand, SOD activity was positively correlated with vitreous VEGF [40].

SOD activity could be important in diabetics with PDR, as indicated elsewhere [40-44]. This study shows that it can be monitored with LPO, as they concomitantly change in both the eye and in serum. For the exact dynamics of oxidative markers and serum-vitreous relations, a multi-year study would be required on the same diabetic patients from early diabetes onset to late retinopathy changes. Nevertheless, studies of the final proliferative state offer reliable initial

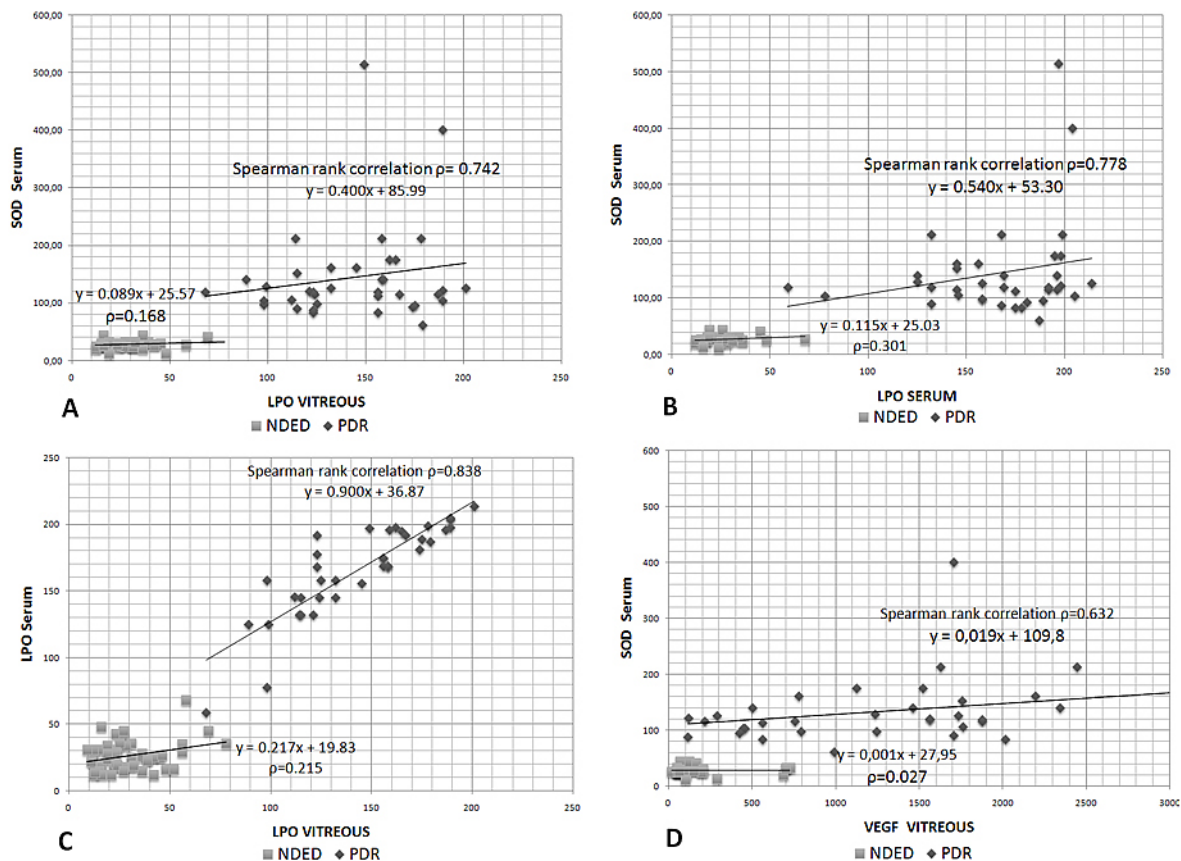


Figure 8. The strongest correlations between the measured markers in non-diabetic patients with eye disorders (NDED) and proliferative diabetic retinopathy (PDR) patients; **A)** LPO vitreous and SOD serum, **B)** LPO serum and SOD serum, **C)** LPO vitreous and LPO serum, **D)** VEGF vitreous and SOD serum.

findings of candidate markers [26,40,45]. Based on the results of this study, it can be concluded that further research on concomitant changes and ratios of vitreal and serum LPO and SOD activity could be promising as possible indicators of oxidative change in the eye, if performed alongside other linked ratios [41,45]. These conclusions are supported by the significant correlations reported in this study. The correlations suggest that monitoring their mutual alterations might be informative during PDR development and should be taken into consideration in further research, including animal studies and human studies of diabetic retinopathy development in asymptomatic patients with no clinical signs.

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