The European Research Journal 2022;8(5):589-597 Original Article

DOI: 10.18621/eurj.1026514 *Endocrinology and Metabolism*

The investigation of thiol-disulfide homeostasis in patients with diabetic peripheral neuropathy

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ABSTRACT

Objectives: Oxidative stress plays a significant role in the pathogenesis of chronic diabetic complications. Hyperglycemia induced oxidative stress is prominent for the development of diabetic polyneuropathy (PNP). Thiol disulfide homeostasis plays a vital role in antioxidant defense. In this study, we aimed to investigate thiol-disulfide homeostasis, total antioxidant capacity (TAC), and advanced oxidant protein products (AOPP) in patients with PNP.

Methods: Eighty patients with T2DM and 19 healthy controls were included in the study. PNP was assessed by using the Michigan Neuropathy Screening Instrument and Electroneuromyography. TAC, AOPP, and total thiols, native thiols and disulfide levels of thiol-disulfide homeostasis parameters were studied with serum samples. The results were compared in patients with/without PNP and control group.

Results: Serum HbA1c (9.5 ± 2.0% vs 8.0 ± 1.8%; $p = 0.019$) and triglyceride levels (204.4 ± 77.0 vs 151.7 ± 58.5 mg/dL, $p = 0.014$) were significantly higher and serum total thiol levels (540.4 ± 9.9 vs 566.7 ± 2.6 µmol/L, $p = 0.038$) were significantly lower in patients with PNP. Serum TAC, AOPP, native thiol, and disulfide levels were comparable among patients with/ without PNP. Serum CRP, AOPP, total thiol, and native thiol levels were found to be higher in patients with type 2 DM ($p = 0.001$, $p = 0.002$, $p = 0.02$ and $p = 0.03$; respectively) compared to the control group. No correlation was observed between serum thiol-disulfide homeostasis parameters and serum glucose and HbA1c levels.

Conclusions: Our study reveals that oxidative stress markers such as serum TAC, AOPP, and disulfide levels are closely related to the existence of diabetes. No significant difference was noted among patients with and without diabetic PNP.

Keywords: Diabetic peripheral neuropathy, oxidative stress, thiol-disulfide homeostasis, total antioxidant capacity, advanced oxidation protein products

Diabetes Mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from insufficient insulin secretion and/or resistance in the body. Previous studies report that oxidative stress,

Received: ***November 24, 2021;*** *Accepted:* ***February 5, 2022;*** *Published Online:* ***July 23, 2022***

How to cite this article: Üstün Eroğlu D, Kıyıcı S, Üstündağ Y, Sığırlı D, Büyükkoyuncu Pekel N, Koca N, et al. The investigation of thiol-disulfide homeostasis in patients with diabetic peripheral neuropathy. Eur Res J 2022;8(5):589-597. DOI: 10.18621/eurj.1026514

e-ISSN: 2149-3189

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imbalance of oxidants, and antioxidants in favor of oxidants in the body, plays a major role in the development, pathogenesis, and progression of chronic complications of DM [1-4].

Diabetic peripheral neuropathy is one of the most common chronic microvascular complications of type 2 DM (T2DM). The pathogenesis of diabetic neuropathy is still unclear. However, currently accepted hypotheses focus on hyperglycemia-induced oxidative stress and their interactions for the development of nerve damage [5].

Proteins, glutathione, homocysteine, and some other molecules contain thiols groups, which are organic compounds that contain functional sulfhydryl groups. Thiol groups may form disulfide bonds under oxidative conditions. These formed disulfide bonds can be reduced back to thiol groups to maintain thiol-disulfide homeostasis. Thiol disulfide homeostasis plays an important role in many physiological processes such as antioxidant defense, protein structures stabilization, and enzymatic activity management [6].

Imbalance in thiol-disulfide homeostasis is thought to play a role in the pathogenesis of DM and obesity [7, 8]. Ates *et al*. [9] assessed the role of thiol-disulfide homeostasis in prediabetic patients in a recent study. Their results suggest that impaired thiol-disulfide homeostasis is important in the development of diabetes and diabetes-related diseases.

Advanced oxidation protein products (AOPP) and total antioxidant capacity (TAC) are also important markers in assessing oxidative stress in diabetic patients [3, 10, 11]. Hyperglycemic state in DM leads to non-enzymatic glycolysis, oxidative and carbonyl stress [3, 10]. The amino acids in the structure of proteins are altered due to oxidative stress and form advanced glycation end products (AGE) and AOPP. The structural alterations in the molecules damage biologically important compounds [10, 11]. AOPP is a cross-linked di-tyrosine protein product and is considered to be a reliable marker for the detection of protein damage [12, 13]. TAC shows the total activity of all enzymatic or non-enzymatic antioxidants [14].

The relationship between DM and thiol-disulfide homeostasis studied in recent studies [15, 16]. To the best of our knowledge, there are no studies that investigated the relationship between diabetic polyneuropathy (PNP) and thiol-disulfide homeostasis.

In the current study, we investigate the relationship between diabetic PNP and thiol-disulfide homeostasis, AOPP, and TAC.

METHODS

Eighty T2DM patients between the ages of 40 and 55 years from the Endocrinology outpatient clinic and 19 healthy volunteers were included in this cross-sectional study. Local Ethical Committee approval was received (02.11.2016 and 2011-KAEK-25 2016/19-03), and informed consent was taken from all patients and healthy participants.

Diagnosis of acute or chronic infectious disease, malignancy, chronic liver disease (AST and ALT ≥ 3×ULN), severe renal insufficiency [glomerular filtration rate (GFR) < 60 ml/min/1.73 m^2], decompensated heart failure, severe cardiac arrhythmia, having a history of acute coronary events within the last six weeks, surgery, burn or severe trauma within the last one month, pregnancy, lactation, smoking and endocrine diseases other than T2DM, hypertension and dyslipidemia were the exclusion criteria for the patients.

The Control group is selected from non-smoker healthy volunteers aged between 40 to 55 years. Any known acute and chronic illnesses, pregnancy, lactation, and usage of antioxidant medication were the exclusion criteria for healthy volunteers.

A detailed physical examination of all participants in the study was performed. Blood pressure was measured using a sphygmomanometer at an upright sitting position after at least 5 minutes of rest. Repeated blood pressure measurements within 2 minutes were obtained by the same physician, and the average of readings was recorded. A platform scale was used for weight measurements. Body mass index (BMI) was computed as weight in kilograms divided by height in meters squared and recorded. The body fat ratio was measured by the bioimpedance method using the Tanita® instrument.

Feldman *et al*.'s [17] two-steps quantitative clinical and electrophysiological assessment was used for the diagnosis and staging of diabetic PNP. The Michigan Neuropathic Screening Instrument (MNSI) and electroneuromyography (EMG) was performed to all

participants with T2DM. Diabetic PNP diagnosis was confirmed in patients with both positive MNSI assessment and EMG results. MNSI, which is used for the assessment of distal symmetrical peripheral neuropathy, includes two separate assessments consist of a 15-item questionnaire and lower extremity physical examination. It is used to assess distal symmetrical peripheral neuropathy in diabetes. EMG study was performed using the Nihon Kohden MEB9102K device. All simulations were performed supra-maximally with bipolar stimulus electrodes. The nerve conduction study was performed in the unilateral upper and lower extremities. Neuro-conduction study protocol includes unilateral studies of sural sensory, ulnar sensory, and median sensory nerves, and peroneal, tibial, median, and ulnar motor nerves with F waves. The minimum case definition criterion for electrodiagnostic confirmation of distal symmetric polyneuropathy is an abnormality ($\geq 99^{th}$ or $\leq 1^{st}$ percentile) of any attribute of nerve conduction in 2 separate nerves, one of which must be the sural nerve 18. T2DM patients were grouped as PNP and without PNP (woPNP) according to MNSI and EMG results.

Laboratory Analysis

Venous blood samples were taken for the measurement of the serum thiol-disulfide homeostasis parameters, TAC, and AOPP levels after 8-12 hours of fasting. Serum samples were centrifuged for 10 minutes at 3000 rpm and stored at -80 ºC. The serum levels of triglyceride (TG), total cholesterol (TChol), HDL-cholesterol (HDL-C), ALT, creatinine and fasting plasma glucose (FPG) were determined using commercially available assay kits with an Olympus AU 2700 auto-analyzer (Olympus Diagnostics, GmbH, Hamburg, Germany). The LDL-cholesterol (LDL-C) was calculated using the Friedewald formula 19. HbA1c level was determined by Adams HA-8160 (Arkray KDK, Shiga, Japan), which uses a cation exchange HPLC method.

Total antioxidant capacity was measured with the ferritic reducing ability of plasma method applied to micro ELISA on a Read well Touch Elisa plate analyzer (Robonik PVT Ltd. Mumbai, India) [20]. The AOPP levels of the samples were determined by the spectrophotometric method developed by Witko-Sarsat *et al*. [21] and defined as µmol/L in chloramine-T equivalents. The thiol/disulfide homeostasis assay was studied according to the method described by Erel and Neşelioğlu [22]. Serum CRP levels were measured by the BN II system nephelometric analyzer (Dade Behring, Germany).

Statistical Analysis

The distribution of continuous data was assessed with the Shapiro-Wilk test of normality. The Mann–Whitney U test or Independent sample t-test was used, when appropriate, to compare differences between the two groups. One-way ANOVA was used to compare more than two independent groups in a normal distribution, and the Bonferroni test was used when significance was found. Kruskal-Wallis test was used for non-normal distribution data, and the Mann-Whitney U test was used in binary comparisons when significant differences were found. Variables are given as mean ± standard deviation. Pearson Chi-square test, Fisher's exact chi-square test, and Fisher-Freeman-Halton test were used for comparison of categorical variables, and data were given with frequency and percentage values. Relations between variables were examined by Spearman's correlation coefficient. $\alpha = 0.05$ was considered as statistically significant. Statistical analyzes were performed in the IBM SPSS Statistics 22 program.

RESULTS

Eighty patients with T2DM (female/male = 46/34) and 19 healthy volunteers (female/male = 15/4) were included in the study. Patients with discordant EMG and MNSI results were excluded, and statistical analysis was performed with 31 patients with PNP, 24 patients woPNP, and 19 healthy volunteers. The demographic characteristics and laboratory findings of the participants are summarized in Table 1.

The mean age of the PNP, woPNP, and control groups were comparable (49.1 ± 4.6 years, 49.3 ± 4.0 years, and 46.5 ± 4.1 years; respectively). There was no significant difference in age, gender, and blood pressure control among the three groups. The mean BMI was significantly higher in PNP group (32.5 ± 7.8 kg/m^2) compared to woPNP (30.8 ± 2.7 kg/m^2) and control group (27.3 ± 4.8 kg/m^2, $p = 0.005$).
While FPG levels were similar (218.1 ± 85.5 mg/dL vs 177.4 ± 67.3 mg/dL, $p = 0.067$), HbA1c levels were

Table 1. Demographics and laboratory results of patients with type 2 diabetes mellitus and controls

	Patients without PNP (n = 24)	Patients with PNP (n = 31)	Controls (n = 19)	*p* value
Age (years)	49.3 ± 4.0	49.1 ± 4.6	46.5 ± 4.1	0.092
Diabetes duration (years)	4.6 ± 3.1	7.2 ± 6.1	-	
Female, n (%)	11(45.8)	19 (61.3)	15 (78.9)	0.087
Male, n (%)	13 (54.2)	12 (38.7)	4 (21.1)	0.080
BMI (kg/m²)	30.8 ± 2.7	32.5 ± 7.8	27.3 ± 4.8	**0.005**
SBP (mmHg)	115.4 ± 8.8	117.7 ± 9.5	107.9 ± 25.7	0.131
DBP (mmHg)	75.0 ± 11.4	75.8 ± 8.8	77.8 ± 10.8	0.655
FPG (mg/dL)	177.4 ± 67.3	218.1 ± 85.5	94.8 ± 9.2	**< 0.001**
HbA1c (%)	8.0 ± 1.8	9.5 ± 2.0	-	
TChol (mg/dL)	218.6 ± 40.5	228.5 ± 45.1	222.3 ± 25.01	0.753
LDL-C (mg/dL)	137.4 ± 30.1	165.3 ± 116.0	138.5 ± 26.3	0.362
TG (mg/dL)	151.7 ± 58.5	204.4 ± 77.0	105.4 ± 52.2	**< 0.001**
HDL-C (mg/dL)	52.0 ± 14.4	45.3 ± 9.3	59.1 ± 16.7	**0.04**
ALT (U/L)	37.2 ± 19.9	37.0 ± 29.4	20.6 ± 9.9	**0.004**
Cr (mg/dL)	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.398
GFR (mL/dk/1.73 m²)	96.3 ± 16.8	96.5 ± 22.7	89.3 ± 14.4	0.248
Total protein (g/dL)	7.2±0.5	7.9±3.5	7.2 ± 0.4	0.921
Albumin (g/dL)	4.5 ± 0.3	4.7 ± 0.2	4.3 ± 0.2	0.212
CRP (mg/dL)	4.29 ± 2.17	5.5 ± 2.97	3.15 ± 0.5	**0.001**
TAC (µmol/L)	946.4 ± 163.6	929.6 ± 231.0	939.5 ± 131.2	0.883
AOPP (µmol/L)	120.3 ± 45.5	122.8 ± 41.5	79.9 ± 35,0	**0.001**
Total Thiol (µmol/L)	566.7 ± 52.6	540.4 ± 39.9	508.4 ± 70.7	**0.001**
Native Thiol (µmol/L)	532.2 ± 43.7	510.4 ± 44.2	470.5 ± 70.9	**0.001**
Disulfide (µmol/L)	17.1 ± 10.20	14.9 ± 8.2	18.9 ± 8.1	0.218

Data are expressed as mean±standard deviation. PNP = Diabetic Peripheric Polyneuropathy, GFR = Glomerular filtration rate, BMI = Body mass index, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, FPG = fasting plasma glucose, LDL-C = Low density Lipoprotein, TG = Triglyceride, HDL-C = High density Lipoprotein, TChol = Total cholesterol, ALT = alanine aminotransferase, Cr = creatinine, HbA1c = hemoglobin A1c, TAC = Total antioxidant capacity, AOPP = Advanced oxidation protein products

significantly higher in PNP group (9.5 ± 2.0 % vs 8.0 ± 1.8 %, $p = 0.019$) (Table 1). Although serum TChol and LDL-C levels were comparable, differences in serum TG levels reach statistical significance between the PNP, woPNP, and control groups (204.4 ± 77.0 mg/dL, 151.7 ± 58.5 mg/dL, and 105.4 ± 52.2 mg/dL; respectively, $p < 0.001$) (Table 1). Intergroup comparison reveals that TG levels were significantly higher in the PNP group compared to woPNP ($p = 0.014$). It was observed that HDL-C levels were lower in the PNP group compared to woPNP, but the difference could not reach statistical significance (45.3 ± 9.3 mg/dL vs. 52.0 ± 14.4 mg/dL, $p = 0.132$). HDL-C levels were found to be significantly lower in the PNP group compared to the control group (45.3 ± 9.3 mg/dL vs. 59.1 ± 16.7 mg/dL, $p < 0.001$) (Table 1). The evaluation of serum CRP level shows a significant difference among the groups ($p = 0.001$) (Table 1).

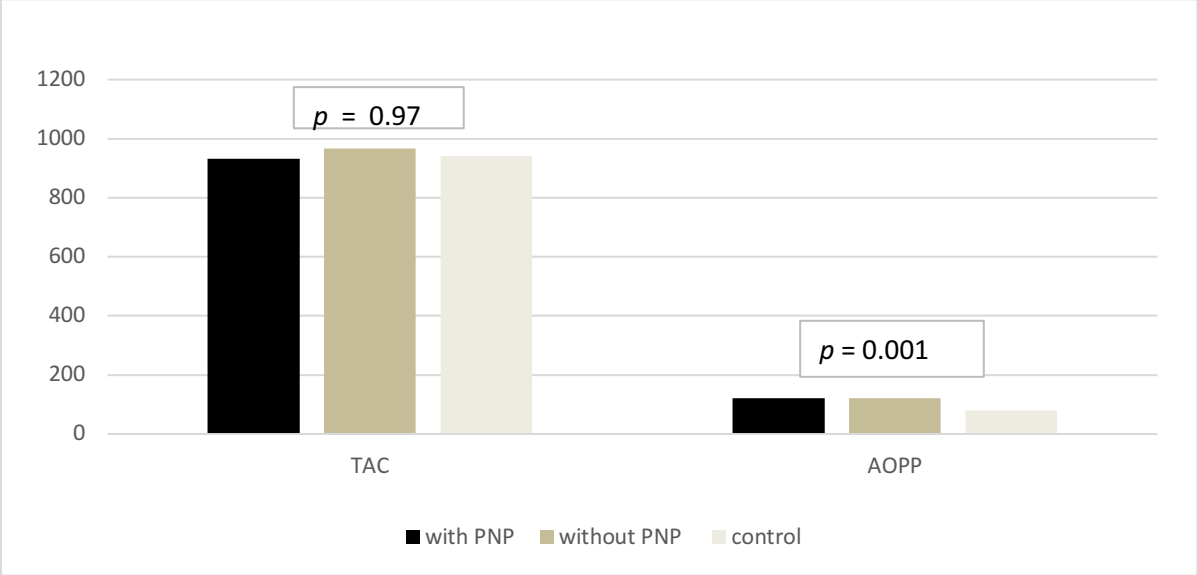

Fig. 1. Serum total antioxidant capacity and advanced oxidation protein products levels of patients with type 2 diabetes mellitus and controls. PNP = Diabetic peripheric polyneuropathy, TAC = Total antioxidant capacity, AOPP = Advanced oxidation protein products.

CRP levels were significantly higher in the PNP and woPNP group than the control group ($p = 0.001$ and $p = 0.02$, respectively).

No significant difference was noted in terms of serum TAC levels among the groups. Serum AOPP level was 122.8 ± 41.5 µmol/L in PNP, 120.3 ± 45.5 µmol/L in woPNP and 79.9 ± 35.0 µmol/L in the control group. While AOPP levels were similar between PNP and woPNP groups, it was higher in PNP and woPNP groups compared to the control group ($p = 0.001$ and $p = 0.002$, respectively) (Fig. 1).

Serum total thiol and native thiol levels were significantly different among the groups ($p = 0.001$) (Table 1). Total thiol levels in woPNP were significantly higher than PNP group (566.7 ± 52.6 µmol/L vs 540.4 ± 39.9 µmol/L, $p = 0.038$) and control group (566.7 ± 52.6 µmol/L vs 508.4 ± 70.7 µmol/L, $p = 0.001$). The native thiol levels were significantly higher in PNP and woPNP groups compared to the control group ($p = 0.004$ and $p = 0.001$; respectively), while no difference was observed between PNP and woPNP group ($p = 0.093$) (Fig. 2).

No significant difference between the serum disulfide levels, disulfide/native thiol ratio, disulfide/total thiol ratio, and native thiol/total thiol ratio when the three groups were compared. No correlation was observed between thiol-disulfide homeostasis parameters and FBG and HbA1c levels.

In terms of medical treatment history, oral antidiabetic drug (OAD) usage was higher in woPNP compared to the PNP group (87.5% vs. 41.9%, $p = 0.003$). Insulin usage with and without OAD treatment (41.9% and 16.1%, respectively) was higher PNP group (4.2% and 13.9%, respectively). The comparison of antihypertensive treatment shows no significant difference between PNP and woPNP group ($p = 0.179$).

DISCUSSION

The development of diabetic neuropathy is closely related to diabetes duration and glycemic control, similar to the other microvascular complications of DM [23]. Studies showed that the duration and severity of hyperglycemia is the most important factor in the development of neuropathy in patients with T2DM [23, 24]. Furthermore, neuropathy development is also shown to be associated with HbA1c, TG levels, BMI, smoking history, and the presence of hypertension [23-25]. In another prospective study, after one year follows up, serum triglyceride levels were found correlated with neuropathy progression independent of glycemic control, diabetes type, and insulin administration [26]. These results consider that classic vascular risk factors are also increasing the chance of diabetic neuropathy development. Consistent with the

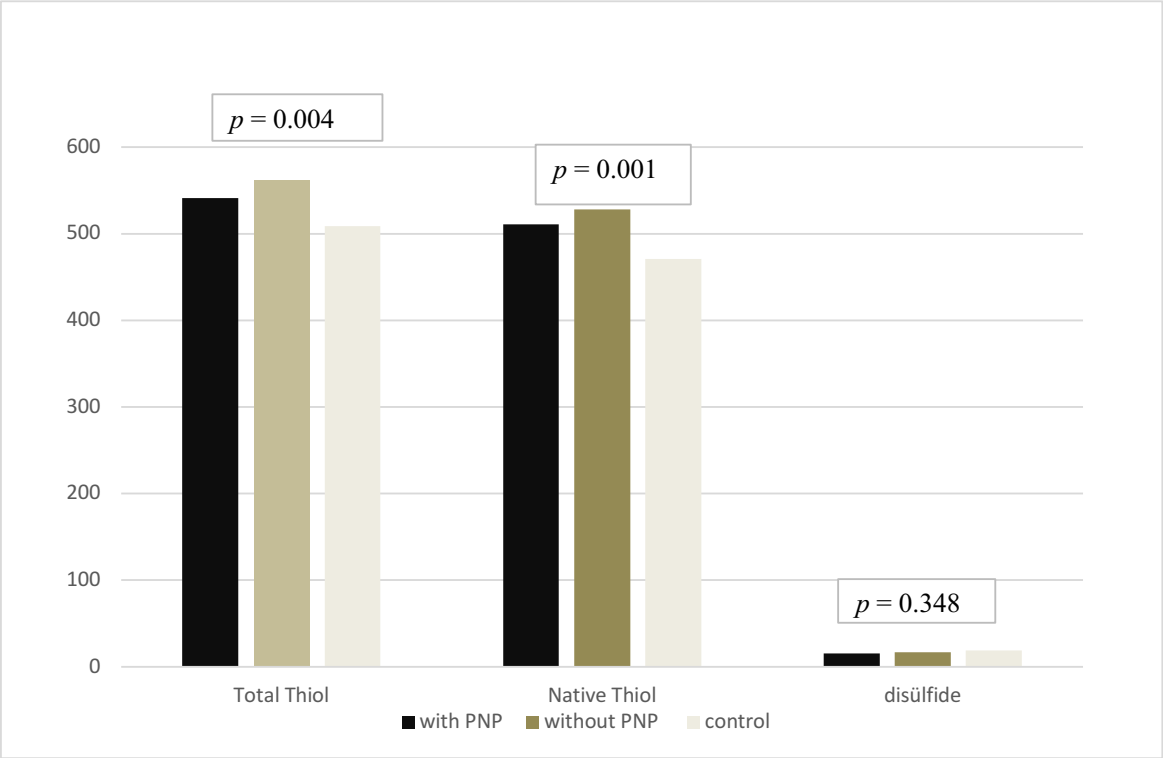

Fig. 2. Thiol-disulfide homeostasis parameters of patients with type 2 diabetes mellitus and controls. PNP = Diabetic peripheric polyneuropathy.

previously published studies, our study also proves that the patients with PNP had worse glycemic control, higher serum TG and lower HDL-C levels. Our results show no relationship between PNP and duration of DM, BMI, and blood pressure measurements.

Dynamic thiol-disulfide homeostasis has shown to contribute to antioxidant protection, detoxification, and apoptosis processes [6-8, 22]. Some molecules, such as proteins, glutathione, and homocysteine, contain thiol groups. Thiols are functional sulfhydryl group, which is oxidized under oxidative conditions and converted to disulfide bonds. The resulting disulfide bonds can be reduced again to the thiol groups, thus attempting to preserve the thiol-disulfide balance. The sum of the existing thiol groups and the reduced thiol groups, also called native thiols, gives the total thiol level. Oxidizing agents in the medium can convert the native thiols to reduced thiol groups, while with the existence of antioxidants, these reduced thiol groups can be converted back to native thiols. Any imbalance in thiol-disulfide homeostasis has also shown to be associated with T2DM and the pathogenesis of obesity [7, 8]. However, there are not enough studies in the literature investigating the relationship between thiol-disulfide homeostasis and diabetic microvascular complications.

Ergin *et al*. [16] showed that patients with T2DM had significantly lower serum total and native thiol levels, higher disulfide, disulfide/native thiol and disulfide/total thiol ratios compared to the control group. Disulfide levels were significantly lower in the newly diagnosed group than the other patients with T2DM. Ateş *et al*. [15] also demonstrated that serum native thiol levels were statistically lower in patients with prediabetes compared to controls. In another study conducted with 30 obese, 27 gestational DM and 68 healthy pregnant women, serum disulfide, disulfide/native thiol and disulfide/total thiol levels increased, and native/total thiols decreased in cord blood of pregnant women with obesity or gestational DM. In addition, the increased levels of disulfide in the cord blood and the reduction of native/total thiol ratio were associated with the poor perinatal outcomes [27]. It has also been reported that serum total and native thiol levels decreased, and disulfide levels increased in patients with type 1 DM [15]. In contrast to these published studies, we observe no significant difference in serum disulfide levels between both patients with and

woPNP and the patients with T2DM and the control group. While native thiol levels were found to be higher in T2DM participants, no significant difference was observed between PNP and woPNP group in our study. The properties of the studied population may have affected our study results. The mean age of the patients was higher, but glycemic control was better in our patients compared to the study population of the Ates *et al*. [15]. Discordant results in our study might be due to the fact that the relationship between PNP and oxidative stress is weaker in advanced age. Moreover, Chakraborty *et al*. [28] showed that metformin treatment restores the altered antioxidant status and inflammatory parameters in patients with T2DM and has antioxidant activity. All of our patients were using metformin treatment, which might explain increased levels of native thiols and decreased disulfide levels in diabetic patients compared with the controls.

Mean serum CRP levels of patients with PNP were found to be significantly increased compared to controls, but no statistical difference was found between the patients with and woPNP in our study. These results may suggest that DM is an inflammatory condition, and the increase of CRP is due to the presence of diabetes rather than diabetic complications. AOPP is an early marker for oxidative stress and is used as a measure of protein damage (predominantly albumin and its aggregates) [13, 21]. Since protein function is strictly dependent on conformation and folding pattern; structural changes in proteins are thought to be among the molecular mechanisms that lead to the complications of diabetes [29]. In addition, the biological effects of AOPPs are similar to those of AGEs and are considered to have a role in inflammatory processes [30]. In our study, mean serum AOPP levels were significantly higher in patients with T2DM. However, there was no difference between those with and woPNP. In our study, HbA1c levels were higher in PNP compared to woPNP, but glycemic control in both groups was not optimal. These results suggest that the AOPP increase is related to poor glycemic control rather than the presence of PNP.

Dordevic *et al*. [31] reported that serum TAC levels, which indicate the total activity of all enzymatic or non-enzymatic antioxidant substances, decreased in patients with T2DM and PNP but serum TAC level did not correlate with blood glucose, diabetes duration, and grade of nerve damage [31]. Although serum TAC level was lower in patients with PNP, no significant difference was noted among the three groups.

Serum CRP, AOPP, total thiol, and native thiol levels were found to be higher in patients with T2DM compared to healthy volunteers. However, no significant difference was observed in terms of serum CRP, AOPP, TAC, native thiols, and disulfide levels between the patients with and woPNP. Although HbA1c levels were significantly higher in PNP group, some studies show that it's possible to occur PNP in prediabetic patients [32].

Limitations

Since our study has a cross-sectional design, there is a need for prospective studies with long-term follow-up and a large number of cases with multiple blood samples taken at different times in order to determine the true role of thiol-disulfide homeostasis in the development of diabetic microvascular complications.

CONCLUSION

These results suggest that oxidative stress parameters assessed in the present study are more closely related to the presence of DM rather than diabetic complications. As a deficiency the example size of the control group is smaller compared to the patient group due to strict exclusion criteria. But, to the best of our knowledge, this is the first study investigates the relationship between dynamic thiol-disulfide homeostasis and PNP.

Highlights

•Serum CRP, AOPP, total thiol, and native thiol levels were found to be higher in patients with T2DM. But the difference between PNP and woPNP group was not significant.

•Oxidative stress parameters assessed in the present study are more closely related to the presence of DM rather than the diabetic complications.

Authors' Contribution

Study Conception: DÜE; Study Design: DÜE; Supervision: SK; Funding: YÜ; Materials: ÖE, NBP; Data Collection and/or Processing: DÜE; Statistical Analysis and/or Data Interpretation: DS; Literature

Review: GE; Manuscript Preparation: DÜE and Critical Review: NK.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Local Ethical Committee (02.11.2016 and 2011-KAEK-25 2016/19-03). Informed consent was taken from all patients and healthy volunteers.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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