Dynamic thiol-disulfide balance and thioredoxin reductase enzyme levels in patients with chronic kidney disease.

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Abstract

Introduction: We aimed to measure the dynamic thiol-disulfide balance and thioredoxin reductase (TrxR) enzyme levels in patients with chronic kidney disease (CKD). Material and Methods: Thirty hemodialysis (HD), 30 CKD patients (stage3-5) and 30 controls were included in the study. The dynamic thiol-disulfide balance was determined by the colorimetric method developed by Erel et al. TrxR levels were determined by ELISA. Results: Native and total thiol levels of CKD and HD patients were significantly lower than that of the control group (p=0.001for both). However, disulfide levels were significantly higher in the HD group (p=0.001), but there was no significant difference between control and CKD groups(p=0.547). A notable negative correlation was found between the native and total thiol levels and IMA(r=-0.628;-0.631),BUN (r=-0.747;-0.747),and creatinine(r=-0.732;-0.721). There was a significant positive correlation between GFR and the thiol levels (r=0.835;0.824). TrxR levels were significantly higher in the patient groups compared to the controls (p=0.001).CRP levels of the patient groups were significantly higher compared to the controls (p=0.001). Conclusions: We have demonstrated that measurement of dynamic thiol-disulfide levels by using colorimetric method can contribute to the diagnosis and follow-up of the disease as a marker, because, it is easily applicable in routine clinical biochemistry laboratories and related with disease severity in CKD patients. Also, we showed that volume correction due to dialysis process should be consider in studies dealing with plasma thiol values and the final results should be given after the correction process.

ABSTRACT

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Conclusion: We have demonstrated that measurement of dynamic thiol-disulfide levels by using colorimetric method can contribute to the diagnosis and follow-up of the disease as a marker, because, it is easily applicable in routine clinical biochemistry laboratories and related with disease severity in CKD patients. Also, we showed that volume correction due to dialysis process should be consider in studies dealing with plasma thiol values and the final results should be given after the correction process.

Keywords: Chronic kidney disease, hemodialysis, oxidative stress, thiol- disulphide homeostasis, thioredoxin reductase

What is known

- CKD is a syndrome characterized by the progressive and irreversible loss of nephrons due to various diseases.
- Oxidative balance is disrupted due to overproduction of free radicals and insufficient antioxidant system in HD patients.
- The main target of free radicals is thiol groups in the sulfur-containing amino acids of proteins.

What's new

Volume correction, which may be caused by this dialysis process, should be taken into account in studies dealing with plasma thiol values and the final results should be given after the correction process.

Measurement of dynamic thiol-disulfide levels by using colorimetric method can contribute to the diagnosis and follow-up of the disease as a marker.

Introduction

Chronic Kidney Disease (CKD), is a syndrome characterized by the progressive and irreversible loss of nephrons due to various diseases [1,2]. CKD has not only significantly increased morbidity and mortality, but also decreases quality of life. The risk of mortality in hemodialysis (HD) patients is about 10 to 20 times higher than in the general population [3]. In these patients, many molecules (e.g. uremic toxins) accumulate in the body which contributes to uremic symptoms and increases mortality [4]. High level of uremic toxins lead to increased oxidative stress in several tissues. Furthermore, increased oxidative stress has negative effects on macromolecules such as lipids, proteins, and nucleic acids [5].

It is well known that oxidative balance is disrupted due to overproduction of free radicals and insufficient antioxidant system in HD patients. Therefore, there are many studies analyzing oxidative stress levels in HD patients [6-8]. Enzymatic and non-enzymatic defense mechanisms against the harmful effects of free radicals are known to exist. One of these antioxidant mechanisms is the presence of thiol-containing compounds. Thiols, also called mercaptans, are sulfhydryl group-containing (-SH) compounds. The main target of free radicals is thiol groups in the sulfur-containing amino acids of proteins. Thiol groups interact with free radicals to form reversible disulfide bonds it then reduced back to thiol groups by several antioxidants. Thus, dynamic thiol-disulfide balance is achieved [9].

Dynamic thiol-disulfide balance has a vital role in the organism and is important to maintain this balance. Thiol-disulfide balance has been measured in only one direction since 1979, but henceforth with novel automated method developed by Erel et al., the level of both variables can be measured distinctly and collectively [10]. In the literature, there are studies related to analysis of thiol levels in CKD patients, but yet there is no study showing the effect of dynamic thiol-disulfide balance and hemodialysis on thioredoxin reductase enzyme levels.

Thioredoxin reductase (TrxR) is a homodimeric flavoenzyme responsible for the catalysis of thioredoxins. TrxR is also of vital importance in controlling intracellular redox medium, cell growth, and apoptosis [11]. Thus, TrxR plays a pivotal role in the pathophysiology of chronic diseases. The sulfhydryl groups of thioredoxins are involved in cellular regulation of various biochemical mechanisms with different functions and the regeneration of inactive proteins as a result of oxidative stress [12]. In this study, we addressed to indicate

the relationship between the dynamic thiol-disulfide balance, systemic oxidative stress parameters and TrxR enzyme levels in CKD (stage 3-5) and HD patients.

Materials and Methods

Study and Control Groups

Thirty HD patients and 30 patients with CKD (stage 3-5) patients, and 30 age and sex- matched healthy control group were included in the study. The mean duration of dialysis in the HD group was 70.1 ± 45.0 months.

Patients with acute and chronic infection, chronic inflammatory disease, hematologic disease, and malignancy were excluded from the study. Demographic informations such as age, gender, duration of dialysis, etc. were collected from the hospital database system. Body mass indices (BMI) of the patient and control groups were calculated using kg/m^2 formula. Hatay Mustafa Kemal University Ethics Committee confirmed the study protocol (protocol number: 23017/128). Informed written consent was obtained from all patients.

Samples collection

Fasting venous blood samples were collected into vacutainer tubes containing EDTA and lithium-heparin from patients with CKD and control subjects. For those patients receiving HD, blood samples were collected before and after the midweek dialysis session. All samples were centrifuged at 1500 x g for 10 min immediately after sampling. Then, after the separation process serum and plasma samples were portioned and stored -80 °C until the time of assay.

Measurement of Biochemical Parameters

Assay Principle of Thiol/Disulfide Homeostasis Parameters

Total thiol and native thiol measurements were performed using Modified Ellman method of Erel et al. [10]. The reagent to be used for total thiol measurement was named 1 (R1) and the reagent to be used for native thiol measurement (R1'). While these initial reagents were different in total thiol and native thiol measurements, the other reagents were the same. R1 was used freshly prepared on the day of the study, with a final concentration of 378 mg of sodium borohydrate (NaBH4) in 1000 mL of water-methanol solution (with a volume ratio of 1/1) of 10 mM. This reducing solution was used to determine the total thiol content. R1' 585 mg of sodium chloride (NaCl) in 1000 mL of water-methanol solution (1/1 volume ratio) was prepared freshly on the day of the study, with a final concentration of 10 mM. The obtained reagent was stored at +4 °C for 6 months. This solution was used to determine the native thiol amount. Reagent 2 (R2) was prepared freshly by dissolving 0.5 mL of formaldehyde with a final concentration of 6.715 mM and 3.8 g of EDTA with a final concentration of 10 mM in 1000 mL of Tris buffer, 100 mM and pH 8.2. The obtained reagent was stored at +4 °C for 6 months. This solution was used for total and native thiol measurements. As to Reagent 3 (R3) 3.963 g of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was prepared freshly on the working day at a final concentration of 10 mM in 1000 mL methanol. This solution was used for total and native thiol measurements.

Total and native thiol measurement principle: For the total thiol amount measurement, 10 µl R1 (10 µl R1 native for native thiol amount measurement) and 10 µl sample were mixed. Then, the first absorbance reading (A1) was performed spectrophotometrically at 415 nm wavelength by adding R2 and R3 (Schimadzu UV-1800 spectrophotometer, Kyoto, Japan). The second absorbance (A2) reading was made at the 10th minute of the reaction at the same wavelength. Absorbance difference (A2-A1) was obtained and the measurement was completed. To determine the total thiol and native thiol levels, the molar extinction coefficient of 5-thio-2-nitrobenzoic acid (TNB) was 14.100 mol / L-1 cm-1. Measurement of disulfide level was calculated using the formula [(total thiol-native thiol) / 2].

We also calculated corrected native thiol, total thiol and disulfide levels based on the serum albumin concentrations from the following formulas:

Corrected total thiol levels: total thiol (μmol/L)/ albumin (g/L).

Corrected native thiol levels: native thiol (μmol/L)/ albumin (g/L).

Corrected disulfide levels: disulfide (μmol/L) /albumin (g/L).

Ischemia-Modified Albumin and Plasma Albumin Levels

We measured Ischemia-Modified Albumin (IMA) levels by using cobalt binding test developed by Bar- Or et al. [13]. Briefly, this method is based on the binding of cobalt with dithiothreitol (DTT) and measuring spectrophotometrically at 480 nm by adding a known amount of cobalt into the serum. The results were expressed as absorbance units (ABSU) (Abbot Architect C-8000). Plasma albumin levels were measured in autoanalyzer by using bromecresol green method (Architect Plus, C-8000, Abbott, USA).

Measurement of Thioredoxin Reductase Levels

We measured serum TrxR levels by using commercial ELISA kit (Bioassay Human TrxR ELISA Kit, Catalog no: E3953Hu). Optical density (OD) on ELISA kit plates was read with a spectrophotometer at 450 nm wavelength (Thermo Scientific- MultiscanGoUV). Working concentrations were calculated using the four parametric logistic (4PL) calibration curve and the values were expressed as ng / mL. The analysis range in the study was 0.05-30 ng / mL and the sensitivity was 0.026 ng / mL. The samples were pre-diluted 8-fold before measurement. The final results were calculated by multiplying with the dilution factor [8].

Statistics

The data were analyzed by using the SPSS 21.0 (IBM, USA) statistical package program. Descriptive statistics for numerical variables were given as mean, standard deviation or, median and minimum and maximum. Descriptive statistics for categorical variables were given as numbers and percentages. Shapiro-Wilk test was used to determine the normal distribution of the groups. For normal distribution data, differences between more than two groups were compared with ANOVA test. For abnormally distributed data, differences between more than two groups were compared with Kruskal-Wallis test. The comparison of parameters before and after dialysis was performed with the paired sample t-test in the normal distribution data and Wilcoxon signed rank questionnaire in abnormally distributed data. Pearson correlation test was used for correlation analysis. Statistical significance level was accepted as $p < 0.05$.

Results

We found that native (-SH) and total thiol (-SH+-S-S-) levels were lower in patients with CKD (stage 3-5) and HD compared with the control subjects (230.7±59.9 and 202.6±79.7 μmol/L; 267.7±66.4, and 264.9±98.6 μmol/L respectively). However, disulfide levels were higher in patients with CKD and HD compared with the control subjects (18.5±7.4 and 31.2±12.3 μmol/L, respectively). Moreover, disulfide levels were significanlty high in the patients receiving HD compared with both patients with CKD (stage 3-5) and control subjects (31.2±12.3μmol/L, 18.5±7.4 μmol/L and 16.3±4.8 μmol/L respectively). Disulfide levels in HD patients increased significantly compared with patients with CKD and control subjects (p=0.001). In addition, plasma IMA levels were significantly different between control and CKD (stage 3-5) and control and HD groups (p = 0.001, Table 1).

Native and total thiol levels showed negative correlation with IMA, blood urea nitrogen (BUN), and creatinine levels (r=-0.628, p=0.001; r=-0.747, p=0.001, r=-0.732, p=0.001). In addition, it showed positive correlation with glomerular filtration rate (GFR) (r= 0.835, p=0.001; r=0.824, p=0.001). There was no correlation between disulfide levels and age and GFR (Table 2). In patients receiving HD, native and total thiols in pre-and post-dialysis were significantly different (p=0.001), but disulfide levels did not significantly change (p=0.0152) by a single dialysis session. Serum albumin and CRP levels were significantly different before and after the dialysis session (p=0.001, Table 3). Therefore, we also calculated adjusted native thiol, total thiol, and disulfide levels based on albumin concentrations in HD patients both before and after the dialysis. After albumin correction, there was no significant difference anymore in the native and total thiol levels

of pre- and post- dialysis patients (p=0.143, p=0.567), however significant difference was observed in the disulfide levels (p=0.001, Table 4).

Discussion

We demonstrated that total and native thiol levels were significantly lower in patients with CKD (stage 3-5) and patients receiving HD than healthy subjects. However, disulfide levels were significantly higher only in patients receiving HD. Moreover, TrxR enzyme levels were significantly higher both in patients with CKD (stage 3-5) and patients receiving HD than healthy subjects. Our study also revealed that IMA and TOS levels were significantly higher in both CKD (stage 3-5) and HD groups compare to controls. However, oxidative stress index (OSI) levels which are calculated by TOS/TAS ratio were significantly higher only in the HD group compare to control.

Coskun et al. showed that the native and total thiol levels of the patients receiving HD treatment were significantly lower than the control group. They hypothesize that low native and total thiol levels occurred as a result of oxidative stress and chronic inflammation in HD patients. In another study, Ates et al. reported that native and total thiol levels were lower in HD patients compared to the control group and they associated this decrease with the reduced total thiol reserves in the organism [11].

In the same line with previous studies, we found that native and total thiol levels in plasma samples of HD patients were lower than both CKD and control groups. Our results were consistent with the literature. One reason of the decrease in plasma thiol levels may be the continuous depletion of sulfhydryl-containing antioxidant molecules, particularly glutathione, to remove ROS as previously suggested [11]. However, although the levels of glutathione as one of the antioxidants are known to be high in the cell, the contribution of other low molecular weight thiol compounds to the plasma sulfhydryl pool is relatively low compared to albumin [14]. Therefore, reduced glutathione levels may not be sufficient to explain the total thiol decrease alone in CKD (stage 3-5) patients.

Another reason contributing to decrease in thiol levels can be explained by the dynamic relationship between albumin and thiol balance. It is known that albumin constitutes most of the thiol pool in plasma (70-90%). Albumin is known to be irreversibly converted into end products as a result of prolonged oxidative damage. One of these albumin-transformed products is sulfenic acid (RSOH), which results in an increase in the presence of oxidant, resulting in sulfinic (RSO_2H) or sulfonic (RSO_3H) acid formation, and these products have been suggested to be removed from the circulation through the liver. We may speculate that uremic toxins cause chronic elevation of oxidative stress in CKD patients, albumin is exposed to a constant oxidative stress.

As a result, albumin may be irreversibly converted and withdrawn from the circulation into oxidation products such as sulfenic, sulfinic, and sulfonic acid as previously shown under long-term oxidative stress in CKD patients. In addition, the liver's depletion of plasma glutathione and sulfhydryl sources due to this increased detoxification metabolism may also contribute to low thiol depletion in plasma [12].

As it is well known, GFR is an important diagnostic and follow-up parameter used in predicting renal function loss in renal diseases and is used in the staging of CKD [4]. In this study, we examined the correlations between thiol groups and GFR and found a positive and strong correlation between both native and total thiol levels and GFR. Plasma native and total thiol levels positively and highly correlated with GFR which suggests that thiols can be used as a test parameter related to disease prognosis in CKD patients.

We also evaluated the effect of HD session on native and total thiol levels and disulfide parameters. Total and native thiol levels of samples measured after dialysis were significantly higher compared to the ones before dialysis. However, there was no significant difference between two groups in terms of disulfide level after the correction with albumin, there was no significant difference between the native and total thiol values. On the other hand, the decrease in disulfide levels of the samples after HD was statistically significant. In other words, single HD session did not have a significant effect on total and native thiols, but resulted in a significant decrease in disulfide levels. We consider that volume correction, which may be caused by dialysis,

may be especially important in comparing thiol values associated with albumin. In addition to that, this decrease in disulfide level may be related to the regeneration of plasma thiol redox status by hemodialysis as stated in the previous studies [10].

In the literature, there are only two studies evaluating the effect of hemodialysis on plasma dynamic thiol balance by using Erel method [10]. In these studies, a correction for a possible volume change due to hemodialysis was not mentioned. It is known that during the HD procedure, different degrees of hemoconcentration can occur in the blood due to volume withdrawal from the patients after HD treatment. The resulting increase in analyte concentrations which was proportional to the volume withdrawn may be an important cause of interference, especially for large molecular weight albumin or albumin-related compounds. In the present study, unlike the previous two studies, we determined albumin levels in blood against a possible hemoconcentration before and after the dialysis. Albumin values were significantly higher in the samples after the dialysis.

TrxR enzyme is a selenoprotein that effectively converts the oxidized thioredoxin protein (Trx) to its reduced form. Therefore, they are responsible for the regeneration of Trx and play a role in maintaining the antioxidant effect. In the present study, increased serum TrxR enzyme levels in CKD patients may be explained by the over-expression of the enzyme to increase the antioxidant effect against increased oxidative stress, as suggested in previous studies. As a result, serum TrxR values were significantly higher in CKD (stage 3-5) and HD group compared to healthy controls. This increase was more prominent in the HD group.

IMA is a modified form of albumin due to oxidative stress. Elevated plasma levels of IMA have been shown in diseases associated with increased oxidative stress, particularly in ischemic heart disease [23,24]. In this study, we found that IMA levels were higher in CKD and HD groups compared to the control. However, there was no significant difference between HD and CKD groups. In the literature, Turedi et al. reported that IMA levels of patients receiving HD were found to be higher compared to healthy controls [25]. In our study, in accordance with the literature, increased IMA levels support the view that increased oxidative stress may lead to albumin modification.

Conclusion

In conclusion, the results of our study show that dynamic thiol disulfide and TrxR enzyme levels play an important role in the pathogenesis of CKD and appear to be associated with oxidative stress. Measurement of dynamic thiol-disulfide levels by using colorimetric method can contribute to the diagnosis and follow-up of the disease as a marker, because, it is easily applicable in routine clinical biochemistry laboratories and related with disease severity in CKD patients. Another result obtained in this study is the remarkable change in thiol values caused by albumin correction. It has shown that volume correction, which may be caused by this dialysis process, should be taken into account in studies dealing with plasma thiol values and the final results should be given after the correction process.

Study Limitations

There are several limitations to this study. One of the limitations is the lack of measuring TrxR enzyme activities. Another limitation is that thiol-containing compounds that contribute to plasma dynamic thiol balance have not been examined separately. Further studies showing the thiol balance in all stages of CKD patients, with a larger population size, will contribute to understanding the role of thiol balance and TrxR enzyme in the pathogenesis of the disease.

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TABLES

Table 1: Thiol-disulfide homeostasis parameters of the study and control groups.

Variables	**Control (n=30)**	**CKD (stage 3-5) (n=30)**	**Hemodialysis (n=30)**	**p**
Native thiol (μmol/L)	463.1 ± 69.1	230.7 ± 59.9	202.6 ± 79.7	0.001^a,0.001^b,0.665^c
Total thiol (μmol/L)	495.7± 68.7	267.7 ± 66.4	264.9 ± 98.6	0.001^a,0.001^b,0.999^c
Disulfide (μmol/L)	16.3 ± 4.8	18.5 ± 7.4	31.2 ± 12.3	0.547^a,0.001^b,0.001^c
Disulfide / Native thiol (%)	3.6± 1.46	8.5± 4.4	20.7 ± 14.6	0.001^a,0.001^b,0.001^c
Disulfide /Total thiol (%)	3.3 ± 1.2	7.0 ± 3.3	13.4 ± 6.5	0.001^a,0.001^b,0.001^c
Native thiol / Total thiol (%)	93.3 ± 2.5	85.7 ± 5.7	73.4 ± 13.1	0.001^a,0.001^b,0.001^c
IMA (ABSU)	0.65 ± 0.06	0.88 ± 0,22	0.91 ± 0.17	0.001^a,0.001^b,0.935^c
TOS (μmol H_2O_2 equiv./lt)	19.4 ± 4.4	27.1 ± 11.2	52.2 ± 24.7	0.004^a,0.001^b,0.001^c
TrxR (ng/mL)	5.2 ± 2.4	7.7 ± 2.1	27.3 ± 19.9	0.001^a,0.001^b,0.001^c
**CRP (mg/L) Median (min-max)	4.02± 1.13 3.1 (3.1;6.0)	6.54± 3.01 5.3 (4.1;15.0)	9.86 ± 5.56 9.6 (1.2;20.9)	0.002^a,0.001^b,0.028^c
ANOVA, **Kruskal Wallis a: Control ve CKD (stage 3-5) ,b: Control and Hemodialysis, c: CKD (stage 3-5) and Hemodialysis	*ANOVA, **Kruskal Wallis a: Control ve CKD (stage 3-5) ,b: Control and Hemodialysis, c: CKD (stage 3-5) and Hemodialysis	*ANOVA, **Kruskal Wallis a: Control ve CKD (stage 3-5) ,b: Control and Hemodialysis, c: CKD (stage 3-5) and Hemodialysis	*ANOVA, **Kruskal Wallis a: Control ve CKD (stage 3-5) ,b: Control and Hemodialysis, c: CKD (stage 3-5) and Hemodialysis	*ANOVA, **Kruskal Wallis a: Control ve CKD (stage 3-5) ,b: Control and Hemodialysis, c: CKD (stage 3-5) and Hemodialysis

Variables		**Age**	**BUN**	**Creatinin**
Native thiol (μmol/L)	**r**	0.166	-0.747	-0.732
	p	0.124	0.001	0.001
Total thiol(μmol/L)	**r**	0.164	-0.747	-0.721
	p	0.126	0.001	0.001
Disulfide (μmol/L)	**r**	-0.057	0.143	0.254
	p	0.603	0.240	0.020
Disulfide/Native thiol (%)	**r**	-0.097	0.597	0.672

Variables		**Age**	**BUN**	**Creatinin**
	p	0.373	0.001	0.001
Disulfide/Total thiol (%)	**r**	-0.098	0.651	-0.682
	p	0.361	0.001	0.001
Native thiol /Total thiol (%)	**r**	0.099	-0.651	-0.725
	p	0.361	0.001	0.001
Pearson corelation test	*Pearson corelation test	*Pearson corelation test	*Pearson corelation test	*Pearson co

Table 2: Correlation analysis of thiol/disulfide homeostasis parameters of the study population

Parametreler	**HD (before dialysis) (n=30)**	**HD (after dialysis) (n=30)**	**p**
Native thiol (µmol/L)	202.6 ± 79.7	305.2 ±78.2	0.001
Total thiol (µmol/L)	264.9 ± 98.6	358.5 ± 83.2	0.002
Disulfide (µmol/L)	31.2± 12.3	26.6 ± 7.71	0.152
Disulfide / Native thiol (%)	20.7 ± 14.6	9.2 ± 3.3	0.001
Disulfide /Total thiol (%)	13.4 ± 6.5	7.65±2.34	0.001
Native thiol/ Total thiol (%)	73.4 ± 13.1	84.7±4.63	0.001
Alb (g/dL)	3.97±0.24	4.97±0.71	0.001
CRP (mg/L)	9.86±5.56	5.47±2.34	0.001
IMA (ABSU)	0.91 ± 0.17	0.87 ± 0.17	0.475
TAS (mmol Trolox equiv./lt)	1.14 ± 0.24	0.65±0.28	0.001
TOS (µmol H_2O_2 equiv./lt)	27.1±11.2	75.1 ± 40.2	0.007
OSI (AU)	2.5 ± 1.1	12.4 ± 8.3	0.001
TrxR (ng/mL)	27.3 ± 19.9	28.3 ± 19.4	0.555
Paired samples t -test	*Paired samples t -test	*Paired samples t -test	*Paired samples t -test

Table 3: Thiol-disulfide parameters in HD patients

Parametreler	**HD (before dialysis) (n=30)**	**HD (after dialysis) (n=30)**	**p**
Corrected Native thiol (µmol/L)	202.6 ± 79.7	244.0± 55.6	0.143
Corrected Total tiyol (µmol/L)	264.9± 98.6	286.5± 56.4	0.567
Corrected Disulfide (µmol/L)	31.2 ± 14.6	21.2± 5.49	0.001
Corrected Disulfide / Native thiol (%)	20.7 ± 14.6	7.42± 2.70	0.001
Corrected Disulfide /Total thiol (%)	13.4 ± 6.5	6.17± 1.95	0.001
Corrected Native thiol /Total thiol (%)	73.4 ± 4.55	68.9 ± 12.4	0.062

Parametreler	**HD (before dialysis) (n=30)**	**HD (after dialysis) (n=30)**	**p**
Paired samples t-test	*Paired samples t-test	*Paired samples t-test	*Paired samples t-test

Table 4: Comparison of albumin corrected results of thiol-disulfide parameters in hemodialysis patients

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`Tables.pdf` available at https://authorea.com/users/407124/articles/517534-dynamic-thiol-disulfide-balance-and-thioredoxin-reductase-enzyme-levels-in-patients-with-chronic-kidney-disease