IJKD KIDNEY DISEASES

Serum Levels of Indoxyl Sulfate and P-cresol in Type II Diabetic Patients With and Without Nephropathy

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Keywords. kidney diseases, indoxyl sulfate, p-cresol, diabetes mellitus type II, diabetic nephropathies

Original Paper

Introduction. Indoxyl sulfate (IS) and para-cresol (p-cresol) are uremic toxins with high protein bonding index that accumulate in the body with decreasing kidney function. The main purpose of the current investigation was to compare the concentration of p-cresol and IS in serum of the type II diabetic individuals with and without nephropathy.

Methods. Fifty-five patients with type II diabetes mellitus were divided into two groups: case and control. The case group consisted of 26 diabetic patients with nephropathy (proteinuria and serum creatinine below 1.5 mg/dL) without any other kidney diseases. The control group included 29 patients without diabetic nephropathy. Patients with advanced heart disease, cerebrovascular accident and other inflammatory or infectious diseases were excluded. Five mL of venous blood was taken from each patient in the morning fasting state. Then other laboratory tests including serum uric acid and creatinine levels, serum urea nitrogen, lipids and glucose were measured by standard methods. P-Cresol and IS levels were measured by the spectrofluorimetric method after extraction. We also filled out a checklist with information regarding the duration of their disease, medication history (oral or injectable), and other demographic information. There were no significant differences between the two groups regarding the investigated factors

Results. There were no significant difference among the investigated factors between the two groups ($P > .05$) except for the serum creatinine, proteinuria and estimated glomerular filtration rate, where the mean values of cases were considerably higher than those of the controls. Serum IS and p-cresol levels were also significantly higher in the case group ($P < .05$).

Conclusion. According to the findings, it seems that IS, and p-cresol may play a role in the development of diabetic nephropathy and other complications of diabetes mellitus.

IJKD 2023;17:126-34
www.ijkd.org
DOI: 10.52547/ijkd.7266

INTRODUCTION

Diabetes mellitus is a global epidemic disease and its prevalence is increasing in developing countries.[1] It is the most frequent cause of kidney diseases and increased cardiovascular mortality.[2] Diabetes mellitus, particularly type II, is becoming more common worldwide. The life expectancy of diabetic patients has significantly increased due

to recent medical advances, leading to increased morbidity and mortality in this patient population.[3]

Diabetic nephropathy (DN) is known as the leading cause of end-stage kidney disease (ESKD).[4] At present, about 40% of patients with diabetes mellitus progress to DN. Although significant improvement has occurred in the diagnosis and management of DN, its molecular mechanism is not known yet.[5] Various conditions including age, genetics, descent, duration of diabetes mellitus, dyslipidemia, hyperglycemia and hypertension, predisposing the individuals to the progression of DN have been reported. However, despite the beneficial effects of controlling blood sugar and blood pressure, the risk of developing DN remains high. Therefore, identifying other contributing factors for the development of DN in a patient may help in determining the disease's risk and management.[6]

Chronic kidney disease is associated with the retention of waste products.[7] When these substances remain in the body, they can alter biochemical and physiological functions which explains the scientific definition of uremic syndrome.[8] Uremic toxins are a group of about 25 different types of toxins with high protein bonding index, with the clinical significance noted in recent clinical studies. Indoxyl sulfate (IS) and para-cresol (p-cresol) are uremic toxins with high protein bonding index that accumulate in the body with decreasing kidney function.[9] Both toxins are derived from amino acids metabolism by the intestinal flora.[10] P-Cresol showed several toxic properties such as inhibition of platelet-activating factor synthesis, decreased response of active polymorphonuclear leukocytes, and decreased response of endothelial cells to inflammatory cytokines in laboratory conditions. Investigations showed that total or free p-cresol concentration is linked to clinical signs in hemodialysis (HD) patients.[11] Observations suggest that high levels of free p-cresol are associated with higher death rates. Increased free p-cresol levels are also a warning sign of cardiovascular disease (CVD) in non-diabetic HD patients.[12]

Recent studies have shown the effects of traditional risk factors including the accumulation of uremic toxins with reduced renal function in the development and worsening of patient's condition.[13] Significant contribution of endothelial dysfunction to the high incidence of cardiovascular disease, which is the leading cause of death among individuals with chronic kidney disease (CKD), has been reported.[14] IS molecules cause increased expression of growth B1-(TGF-B1) factor, tissue metalloproteinase 1 inhibitor (TIMP-1), and inhibitor plasminogen activator 1 (PAI-1), accelerating the progression of renal disease, glomerular sclerosis and interstitial and tubular fibrosis.[14] IS has a negative impact on the growth of smooth muscle and endothelial cells.[15] Previous studies showed that increased concentration of IS is associated with atherosclerosis and peripheral arterial disease in HD and CKD patients.[16] Due to the importance of this issue and the lack of complete and comprehensive information in this area, this investigation aimed to compare the amount of endogenous nitrogen waste products; IS and p-cresol, in type II diabetic cases with and without DN.

MATERIALS AND METHODS

Study Design and Participants

This work is a case-control study that was conducted in a university hospital between 2018 and 2020. The ethics committee of Tabriz University of Medical Sciences (license code: IR.TBZMED.REC.1398.778) approved the study. Fifty-five patients with type II diabetes mellitus at the age range of 45 to 85 years (mean age of 61.8 ± 8.0) with at least five years history of diabetes mellitus were included in this study. The patients' smoking status were not recorded, so its effect was not considered in this study. The case group included 26 patients with type II diabetes mellitus and DN with proteinuria and serum creatinine below 1.5 mg/dL. The control group included 29 diabetic patients without DN. Patients with advanced heart disease, cerebrovascular accidents, and other inflammatory or infectious diseases were excluded from the study. Previously prepared informed consent forms were granted by all cases and controls included in this study.

Laboratory Tests

Five mL of venous blood were taken from all patients in the morning in fasting state and the samples were stored in the refrigerator. The first laboratory tests including fasting blood sugar (FBS), 2 hours post-prandial blood sugar (2hppBS), serum urea nitrogen (SUN), triglyceride, high-density lipoprotein (HDL), serum creatinine, uric

acid, white blood cell (WBC) count, hemoglobin, cholesterol, hematocrit, and hemoglobin A1c (HbA1c) were measured by standard methods. Then the remaining of the serum was stored in a deep freezer (-80 °C). After completing sample collection, serum concentrations of IS and p-cresol were determined by a fluorescence method. A liquid-liquid extraction method, i.e., salt assisted liquid-liquid extraction (SALLE) was employed for the extraction of p-cresol from patients' serum samples. Briefly, about 100 µL of serum samples were mixed with 25 µL of HCl (6 M) and then heated for about 2 min at 80 °C for hydrolysis of p-cresyl glucuronide and p-cresyl sulfate (PCS) to p-cresol (25). Then, about 900 µL of acetonitrile was added to the room temperature cooled samples and shaken for 15 minutes. After then, about 500 µL of NaCl (saturated) was added to the mixture and shaken for about 15 minutes. Finally, the mixture of NaCl solution and serum was centrifuged, and the upper liquid was collected for recoding fluorescence emissions at 310 nm wavelengths with an excitation wavelength of 280 nm.[17] A similar method was also used for the extraction and measurement of IS in serum samples. To precipitate the proteins of serum samples, 100 µL of the samples were mixed with 900 µL acetonitrile. After being vigorously shaken, the proteins were separated by using a centrifuge (10 min, 8000 rpm). Next, 500 µL of NaCl (5 M) solution was added to the 900 µL of the supernatant liquid and was shaken for performing SALLE. After then, the supernatant liquid phase was collected to a microcuvette to determine the fluorescence intensity ($\lambda_{ex}/\lambda_{em}$ = 280 nm/365 nm), and accordingly the concentration of IS in patients' serum samples.[18] Prior to determining IS and p-cresol with the previously constructed methods, their analytical performances were re-checked. Some important validation tests such as linear range, the limit of detection, repeatability (intraday and interday) etc. were also checked to assure the precision and accuracy of the IS and p-cresol detection in serum samples. The measurement method of albuminuria was immune-turbidimetry. Albumin concentrations of 24-h urine samples were determined by using BromoCresol Green (BCG) colorimetric assay. Furthermore, urine samples of the patients were not cultured in this study.

Statistical Analysis

The software used for data analysis was Statistical Package for the Social Sciences (SPSS, version 15) and descriptive statistics (mean and standard deviation (SD)) were employed to describe the variables. Also, t-test for quantitative analysis and chi-square (χ^2) test for qualitative analysis of the obtained data were used. Significance level (*P*) was considered less than .05.

RESULTS

In this study, fifty-five patients consisting of 28 females and 27 males were studied. The case group included15 males and 11 females with a mean age of 62.5 ± 8.2 years, and the control group included 12 males and 17 females with a mean age of 61.2 ± 8.0 years. There was no significant difference between the two groups regarding the mean of age (t-test, $P > .05$). Also, there was no significant difference in terms of gender distribution of the patients in the two groups (chi-square, $P > .05$). The average of body mass index (BMI) was calculated as 29.8 ± 5.2 and 28.3 ± 4.6 for case and control groups, respectively, without a significant difference ($P > .05$). The history of diabetes mellitus in the case group (13.1 ± 6.1 years) was not significantly different with that of the control group (11.1 ± 4.6 years) as showed by the results of a t-test ($P > .05$). Concerning the therapeutic regimen, seventeen patients with diabetic nephropathy and 11 patients without nephropathy were treated with insulin. Twenty-seven of 29 patients with diabetic nephropathy and 14 of 29 in non-nephropathy cases received angiotensin converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs). The results revealed no significant difference between the two groups ($P > .05$) in mean blood pressure or heart disease history. There was no significant difference in the relative frequency of insulin consumption between the case group (57.7%) and the control group (37.9%). The mean value of creatinine in the case group was more than that of the control group, while the mean value of the estimated glomerular filtration (eGFR) in the case group was considerably less than that of the control group. The mean IS level was 33.4 ± 3.3 mg/L in the case group and 10.5 ± 9.0 mg/L in the control group that was significantly higher in DN patients ($P < .05$). The mean of

serum p-cresol level was higher than the control group in DN patients ($P < .05$). There were also significant relationships between serum levels of p-cresol with SUN (R = 0.639), BMI (R = 0.573), eGFR (R = -0.503) and creatinine (R = 0.477) in the case group. Figure 1 illustrates the observed significant correlations in the case group. Serum p-cresol was significantly correlated with eGFR

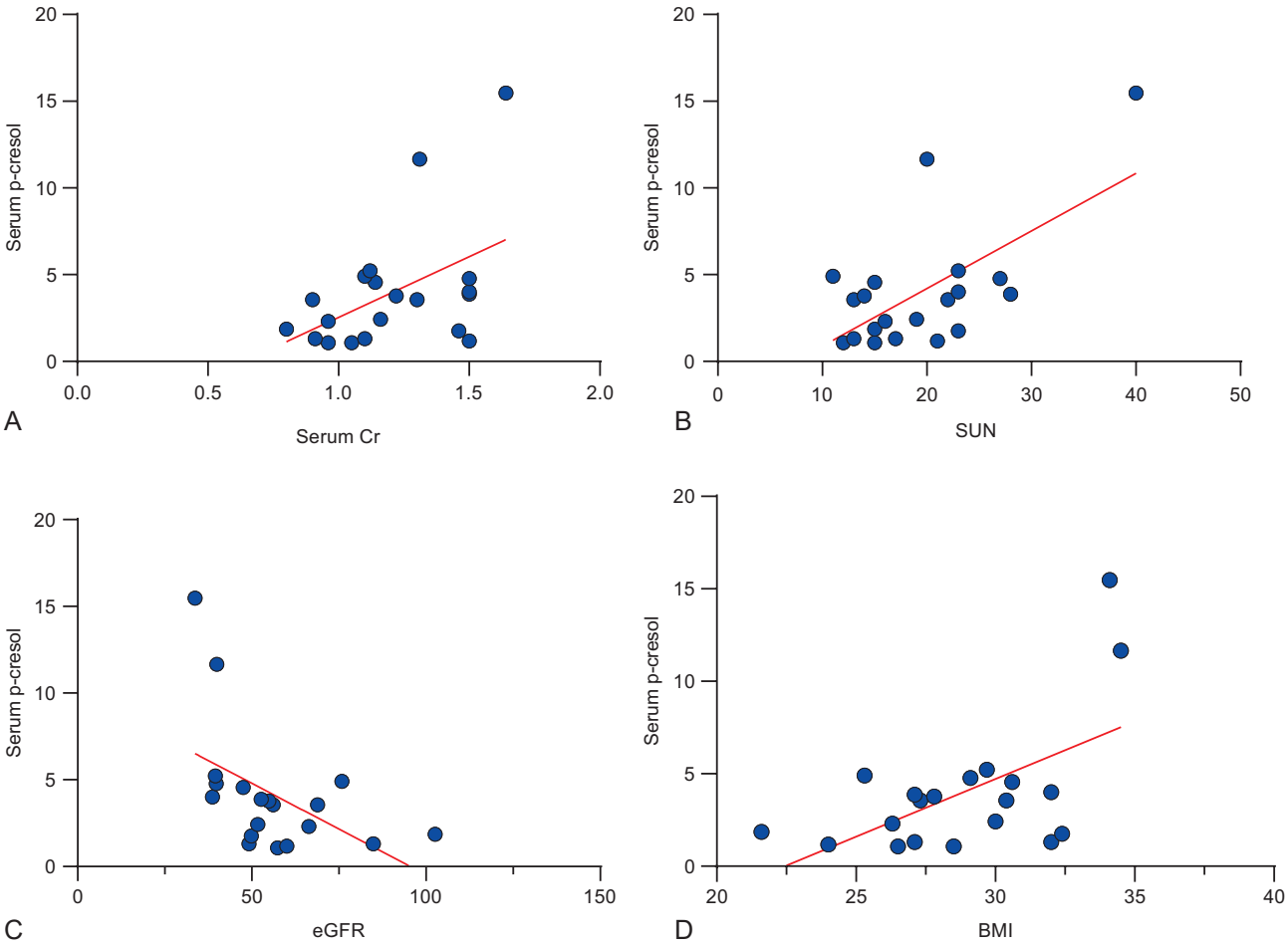

Figure 1. Significant correlations of serum p-cresol (A) serum creatinine (Cr); (B) serum unbound nitrogen (SUN); (C) estimated glomerular filtration (eGFR) and (D) body mass index (BMI)) in the case group.

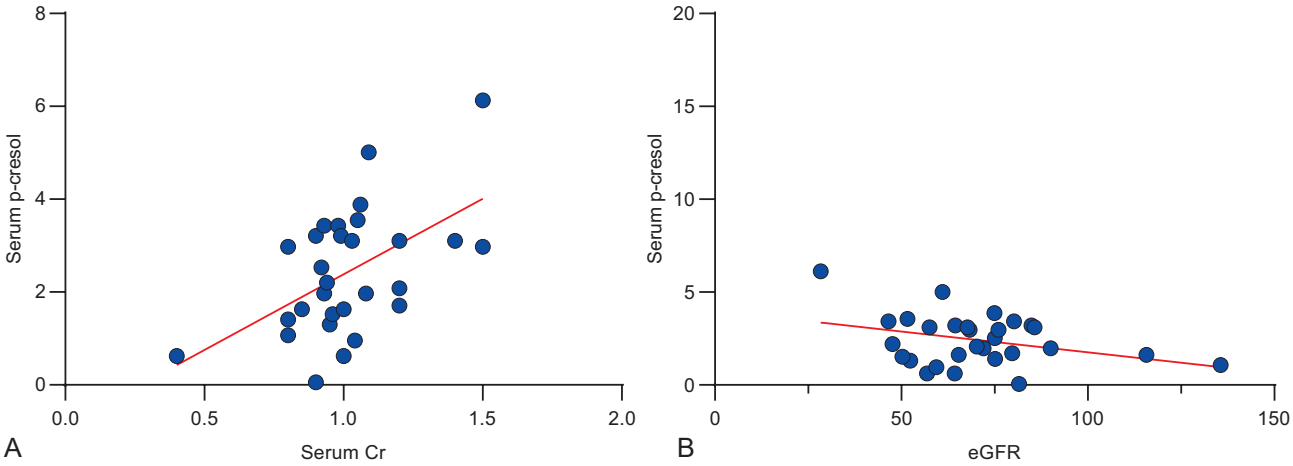

Figure 2. Significant correlations of serum p-cresol (A) serum creatinine (Cr) and (B) estimated glomerular filtration (eGFR)) in the control group.

(R = -0.411) and creatinine (R = 0.535) in the control group. No significant correlation was observed with any of the investigated biochemical parameters and serum IS levels in the control group, except for HDL (R = -0.522). Figure 2 shows the observed significant correlations in the control group. Cockroft-gault (CG), chronic kidney disease epidemiology collaboration (CKD-EPI), and modification of diet in renal disease (MDRD) were utilized to calculate the GFR values.[19] Table 1 lists the GFR of the cases and controls using different formulas. The obtained results showed that there is significant difference between the case and the control groups for all equations. However, CG formula provided a more general view on the creatinine clearance.

The evaluation of laboratory data of the studied groups revealed that the duration of diabetes mellitus in patients with and without DN was not statistically significant ($P > .05$). The other studied parameters including BMI, CBC, FBS, HbA1c, WBC, hemoglobin, hematocrit, SUN, uric acid, TG, cholesterol and HDL were not significantly differed between patients in both case and control groups ($P > .05$). Full detailed data of each group are listed in Table 2.

DISCUSSION

In the present study, the serum concentrations of IS and p-cresol in patients with type II diabetes mellitus with or without diabetic nephropathy were investigated. In the following section, some of the reported approaches for the determination of p-cresol and IS are discussed. Korytowska *et al*.[20] investigated salivary and serum concentrations of IS and p-cresol sulfate in kidney transplant recipients. The reported median serum values for p-cresol sulfate were 7.103, 4.697, and 4.755 mg/L; respectively, for samples collected 1, 3, and 6 months after transplantation. The median serum p-cresol values for case and control groups were 3.55 and 2.20 mg/L with the corresponding IS values of 2.075, 1.715, and 1.870 mg/L; respectively. The median IS values in our study for case and control groups were 32.87 and 8.15 mg/L. Korytowska *et al*.[20] determined p-cresol sulfate and IS concentrations by liquid chromatographic mass spectrometry (LC-MS/MS) method after protein precipitation with methanol. There is no shadow of doubt that LC-MS/MS method provides much more accurate measures compared to simple and low-cost spectrofluorimetric methods, however, the cost of analysis is an important issue in practical

Table 1. Calculation of GFR by Different Formula

Formula*	Gender	Criteria	Equation	Case	Control
CG	Male/Female	-	$eGFR = \frac{72(140 - age)}{Weight \times Creatinine}$ $eGFR = 0.85\left[\frac{72(140 - age)}{Weight \times Creatinine}\right]$	58.3 ± 21.1	70.3 ± 20.8
CKD-EPI	Female	Cr ≤ 0.7	$eGFR = 144 \times \left(\frac{Cr}{0.7}\right)^{-0.329} \times (0.993)^{age}$	62.42 ± 16.14	69.76 ± 16.29
	Female	Cr > 0.7	$eGFR = 144 \times \left(\frac{Cr}{0.7}\right)^{-1.209} \times (0.993)^{age}$		
	Male	Cr ≤ 0.9	$eGFR = 141 \times \left(\frac{Cr}{0.9}\right)^{-0.411} \times (0.993)^{age}$		
	Male	Cr > 0.9	$eGFR = 141 \times \left(\frac{Cr}{0.9}\right)^{-1.209} \times (0.993)^{age}$		
MDRD	Male	For GFR < 60 mL/min/1.73m^2	$GFR = 175 \times (Cr)^{-1.154} \times (age)^{-0.203}$	49.40 ± 5.56	51.45 ± 5.48
	Female	For GFR < 60 mL/min/1.73m^2	$GFR = 175 \times (Cr)^{-1.154} \times (age)^{-0.203} \times 0.742$		

Abbreviations: CG, cockroft- gault; CKD-EPI, chronic kidney disease epidemiology collaboration; MDRD, modification of diet in renal disease

Table 2. Demographic Data and Laboratory findings of the studied patients

Parameters (mean ± SD (n))	Case	Control	*P*
Age, y	62.5 ± 8.2	61.2 ± 8.0	> .54
Weight, kg	83.4 ± 14.2	78.0 ± 13.3	> .15
Sex			
Female	11	17	> .29
Male	15	12	
BMI, kg/m^2	29.8 ± 5.2	28.3 ± 4.6	> .28
Duration of Being Diabetic, y	13.1 ± 6.1	11.1 ± 4.6	> .19
History of Previous Disease			
Hypertension	23	22	> .53
Heart Disease	2	2	
Insulin Consumption			
Consumer	15	11	> .18
Non-consumer	11	18	
WBC	8314 ± 1954 (25)	7709 ± 1637 (29)	> .22
Hemoglobin, g/dL	13.83 ± 1.78 (25)	13.24 ± 1.21 (29)	> .16
Hematocrit (%)	40.92 ± 5.82 (25)	39.87 ± 3.58 (29)	> .42
Serum Unbound Nitrogen (SUN), mg/dL	18.73 ± 6.25 (25)	16.79 ± 4.52 (29)	> .19
Creatinine, mg/dL	1.16 ± 0.26 (26)	1.01 ± 0.21 (29)	> .03
Uric Acid, mg/dL	5.27 ± 0.99 (18)	5.24 ± 0.91 (28)	> .91
FBS, mg/dL	135.8 ± 57.8 (26)	140.9 ± 64.3 (29)	> .76
Two hours post-prandial BS (2hppBS), mg/dL	231.3 ± 99.3 (26)	206.7 ± 89.9 (29)	> .40
Hemoglobin A1c (HbA1c), mg/dL	7.98 ± 1.41 (26)	7.64 ± 1.78 (29)	> .44
Triglyceride, mg/dL	154.27 ± 68.20 (26)	149.38 ± 71.68 (29)	> .80
Cholesterol, mg/dL	165.7 ± 78.6 (26)	149.0 ± 32.2 (29)	> .30
HDL, mg/dL	43.19 ± 20.42 (26)	43.24 ± 7.99 (29)	> .99
Proteinuria			
Micro	26 (86%)	0 (0%)	0.00
Macro	4 (14%)	0 (0%)	
P-Cresol, mg/L	3.98 ± 3.61 (20)	2.45 ± 1.31 (29)	> .04
Indoxyl Sulfate, mg/L	33.38 ± 3.27 (16)	10.46 ± 9.00 (27)	> .01

applications of biomarker and drug assays.[21] In two papers reported by Meijers *et al.*, serum *p*-cresol levels were considerably higher in patients with diabetes mellitus.[22] Despite proper control of known risk factors associated with uremia, progression of kidney dysfunction is still inevitable in a significant proportion of patients. A previous study reported that increasing serum p-cresol is related to the progression of renal disease, and this parameter is independent of other known risk factors including diabetes mellitus, age, malnutrition-inflammation, calcification, and anemia.[23] Our study showed that the level of p-cresol and IS were high in patients with DN, and this is the first study addressing the comparison of gut-derived nitrogenous products in diabetic patients with and without DN.

Meigers *et al.* determined that total serum concentrations of p-cresol and IS, despite their strong correlation with each other in patients with kidney diseases, may not change accordingly and different metabolic pathways may determine the total serum concentrations of these two substances.[24] Despite advances in medical care, mortality of hemodialysis (HD) patient is a threatening clinical challenge. Cardiovascular diseases (CVD) account for almost 50% of all deaths in this group of patients. In short, reliable biomarkers are essential for determining the prognosis of HD patients.[25]

Wang *et al.*[26] reported negative correlations between serum IS levels and HDL-c (R = -0.244) and eGFR (R = -0.245) and a positive correlation with serum creatinine (R = 0.222). Selim *et al.*[27] reported a negative correlation of eGFR with free serum IS (R = -0.526) and total serum IS (R = -0.534) and positive correlations of blood urea nitrogen (BUN) with free serum IS (R = 0.254) and total serum IS (R = 0.236). Their observed inter-correlation among free and total serum IS' R was 0.920.

P-cresyl sulfate (PCS) has been suggested in previous studies as an emerging biomarker in

patients with CKD. High serum PCS concentrations can also predict the prognosis of CVD in HD patients.[7] Reported studies have showed that high serum concentrations of PCS are related to the progression of kidney disease and death in patients with CKD.[9] However, Lane *et al.* showed that there was no correlation between serum PCS level and mortality. Probable reasons for this discrepancy may include short follow-up time, age, and small sample size of the study.[28]

Liabeuf *et al.* have shown that serum PCS is the predictor of death in patients with different stages of CKD.[29] Wang *et al.* reported that high serum PCS concentrations were independently related to the increased risk of mortality and cardiovascular involvement in elderly HD patients.[30] This association with other known risk factors, such as age (at the beginning of the study), malnutrition, anemia, calcium-phosphate imbalance, and diabetes status was not observed. In our study, none of the patients died during the study; however, we did not follow up our patients after completing the study, which is recommended for future studies. Tayebi Khosroshahi *et al.* studied the effect of fermentable high fiber diet supplementation on the p-cresol and IS concentrations in HD patients. p-Cresol and IS levels were determined by using the HPLC-FL method which was validated in plasma. The obtained results showed that p-cresol values ranged from 2.29 to 15.67 mg/L and 3.01 to 11.57 mg/L, while IS values were in the range of 24.32 to 49.57 mg/L and 28.64 to 50.07 mg/L for HD after and before receiving a diet containing resistant starch (HAM-RS2), respectively. The obtained data points for p-cresol are consistent with our reported values. This deviation could be affected by the type of disease and its stage.[31]

Our small-scale study showed the significant role of increased IS levels in the CKD patients. There are some restrictions on generalizing the data, such as different ethnic groups, single-center experiences, observation time, and the unavailability of a free form of toxin measurement. The association between free and/or total solute concentrations, mortality, and CVD in patients with kidney diseases, including nephropathy, has been proved by previous studies.[22,32] However, our study is the first study in which these changes were examined in diabetic patients with nephropathy who have not started dialysis yet.

The results of this study revealed increased levels of p-cresol in diabetic patients with nephropathy compared to a corresponding mean value in diabetic patients without nephropathy. This study has some limitations. The sample size of our study was small, and an assessment of intestinal microbiota was not performed. Lack of follow up was the other limitation of our study. In addition, smoking status of patient was not recorded, so its effect was not addressed in this study.

CONCLUSION

According to the findings of the current study, it seems that nitrogenous waste products such as IS may play a role in the development of diabetic nephropathy and other complications of diabetes mellitus. Serum levels of p-cresol are not connected to IS, which shows different metabolic pathways for each of them. P-cresol and IS are both accurate markers for monitoring the behavior of protein-bound substances in patients with kidney involvement. Serum IS and p-cresol levels may be helpful in the prediction of the risk of kidney failure progression in patients with DN along with other diagnostic methods for kidney function. Further investigation is needed to clarify the mechanisms of the effect of IS and p-cresol on kidney failure progression and to design treatment approaches aimed at reducing these substances.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENT

The authors would like to express their appreciation to all participants and staff for their kind cooperation. This project was support by Tabriz University of Medical Sciences, Tabriz, Iran, under grant # 63218.

ETHICAL CONSIDERATIONS

This study was reviewed and approved by the ethics committee of Tabriz University of Medical Sciences (license code: IR.TBZMED.REC.1398.778).

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Received January 2023
Revised March 2023
Accepted April 2023