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A multivariate generalized linear model of the effect of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1 in end-stage renal disease

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ABSTRACT

End-stage renal disease (ESRD) is a serious disease that requires hemodialysis as the main regimen for supporting patients. The pathophysiology of the disease is still an interesting field of study. In the present study, matrix metalloproteinase-3 (MMP3), a tissue inhibitor of metalloproteinase-1 (TIMP1), and vitamin D (VitD) were studied in ESRD. MMP3, TIMP1, VitD, and other related biomarkers were measured in 53 ESRD patients and compared with 33 healthy controls. The results showed a significant increase in serum MMP3 and TIMP1 and a significant decrease in serum VitD in ESRD patients compared with the control group. The multivariate GLM results revealed no significant effects of the covariates (sex, age, smoking and BMI) on the levels of MMP3, TIMP1 and VitD. ESRD was found to be the major factor affecting the measured parameters, with a huge effect size (Partial η^2) of 0.824. According to our data, eGFR, Creatinine, Urea, phosphate, uric acid and VitD (as estimated glomerular filtration rate) are the top 6 factors highly affected by the presence of the disease. MMP3 and TIMP1 are important parameters in ESRD and may act as drug targets. As the patients also suffered from hypovitaminosis D and hypocalcemia, these factors also need intervention.

INTRODUCTION

Chronic kidney disease (CKD) affects almost 10% of the world's population [1]. When CKD progresses into end-stage renal disease (ESRD), which affects approximately 745,000 individuals in the United States alone, renal replacement therapy via dialysis or kidney transplantation should be considered for management [2]. ESRD is diagnosed when the glomerular filtration rate (GFR) declines to less than 15 ml/min/1.73 m^2 of body surface area (at 15 ml/min/1.73 m^2 of body surface area, kidney failure is regarded) [3]. Various cytokines [4] have been investigated in the ESRD, as have trace metals like copper and zinc [5], as well as oxidative stress biomarkers [6]. Endothelial dysfunction, oxidative stress and inflammation are plasma biomarkers that predict cardiovascular deterioration in CKD [7] indicating CKD progression and cardiovascular decline.

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In addition to metabolic problems, ESRD is characterized by fluid, electrolyte and hormone imbalances, and eventually progresses to uremia [8]. Chronic metabolic acidosis, hyperkalemia, bone degeneration, hyperphosphatemia, edema and irregular blood pressure are prevalent disturbances in ESRD patients. Through careful monitoring of potassium, calcium, protein, sodium and phosphorus, these risks may be reduced, and the disease's course can be controlled, hence alleviating CKD patients' symptoms [9]. Beyond the aforementioned, calcium-phosphate homeostasis and bone metabolism are altered in ESRD patients [10]. Vitamin D (VitD) functions in the body through both an endocrine mechanism (regulation of calcium absorption) and an autocrine mechanism (facilitation of gene expression) [11].

In patients with ESRD, the endocrine mechanism is effectively disabled; however, the autocrine mechanism can function normally as long as the patient has adequate serum levels of VitD, depending on its function [11]. Elevated PTH is a non-specific test that may indicate secondary or tertiary

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hyperparathyroidism reflecting alterations in VitD production and calcium and phosphate homeostasis [12]. One of the most important reported functions of VitD is to promote innate and adaptive immunity, and its seasonal variation has been linked to mortality [13]. ESRD patients are vulnerable to VitD deficiency due to impaired renal hydroxylation, low dietary intake and inadequate sun exposure.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in the degradation of the extracellular matrix, and have been shown to play a major role in extracellular matrix remodeling [14]. MMP3 can degrade many components of basement membranes and connective tissue, such as collagen, fibronectin, proteoglycans and elastin [15]. MMP3 can also activate other metalloproteinases and pro-inflammatory mediators. This enzyme was identified in the kidney's glomerular and tubular cells [16]. Accordingly, MMP3 has been negatively linked with mesangial enlargement and glomerular damage, where its effect is seen as tubular atrophy and interstitial lesions [17]. It has been shown that MMPs activities are regulated by tissue inhibitors of metalloproteinases (TIMPs), which bind to the active sites of MMPs [18].

TIMP1 is a multifunctional protein that acts especially to inhibit MMPs [19]. TIMP1 has been shown to exert an anti-angiogenic activity, both in vitro and in vivo [20]. Patients with diabetes demonstrate increased serum and urine concentrations of TIMP1, which is related to larger glomerular lesions [21]. In the same research that found an increase in MMP3 expression, an increase in TIMP1 expression was seen in tubules with atrophy; however, both increases were negatively linked with established glomerular mesangial expansion [17]. Human glomeruli express TIMP1 and TIMP-2, and the upregulation of both, has been demonstrated in glomerulosclerosis [22]. However, in human renal biopsies manifesting focal segmental glomerulosclerosis, a marked overproduction of TIMP1 was reported [23]. These results and the results of our study indicate a difference in the mechanisms of focal segmental glomerulosclerosis and ESRD. Susceptibility, according to [23], is revealed very early and is associated with early growth response factor-2 *in vivo* and *in vitro*. In our study, we observed similar expression patterns

The dysregulation of MMP/TIMP has been documented in clinical research conducted on CKD patients [24]. In patients with CKD, increasing glomerular lesions have been associated with reductions in serum TIMP1 and TIMP-2 levels, and these parameters can be increased by renin-angiotensin system blockade [24]. In the present study, the correlation between the electrolyte levels and MMP3 and its inhibitor (TIMP1) are examined so as to obtain an idea about the possible interaction between these parameters in ESRD patients.

SUBJECTS AND METHODS

Subjects

This study involved sixty ESRD patients (28 male and 29 female) aged (46.26±10.31 years). All had a history of AKI that progressed to renal failure, and all were on continuous dialysis. AKI in the case of these patients was caused by severe prolonged infection, hemolytic anemia, prolonged blockage of ureters, and undefined causes of renal failure. We excluded AKI cases caused by heart (cardiovascular) diseases or lupus erythematosus, as well as via diabetes and liver disease.

The patients were recruited between December 2021 and February 2022 at the Dialysis Unit of Al-Hakeem General Hospital and Al-Sadr Medical City in the Governorate of Najaf, Iraq. Patients were evaluated based on a comprehensive medical history that considered the presence of systemic disease. The duration of HD was 3.90±1.58 years. All participants had serum C-reactive protein (CRP) concentrations of less than 6 mg/L (as measured by the agglutination test). The test was conducted to rule out overt inflammation, which causes alterations in acute-phase reactants. According to procedures listed under the 10th revision of the International Statistical Classification of Diseases and Related Health Problems, a senior physician provided a diagnosis (2021 ICD-10-CM Diagnosis Code N18.6). All patients received the following treatments: folic acid, iron, calcium carbonate, Eprex and heparin.

Thirty-three healthy individuals (11 females and 22 males) without apparent physical illnesses formed the control. Their age range was 46.91±6.81 years old, which matched that of the patients. All participants (as patients or through first-degree relatives) gave informed written consent. The Iraqi institutional review board approved the protocol of the University of Kufa (622/2021), Kufa, Iraq.

Measurements

Venous blood samples were taken from the participants between 8.00 a.m. and 9.00 a.m. after 12 hours of fasting. Venous blood samples were collected into plain tubes. Samples were aliquoted and stored at -80°C before assay. The serum was sampled directly before the hemodialysis session to assay all parameters. After separation, the sera were distributed into three new Eppendorf® tubes for further analysis. Serum MMP3, TIMP1 and VitD concentrations were measured using ELISA kits supplied by Melsin Medical Co., Ltd., Jilin, China. The inter-assay CV% of all the kits was <10% and the sensitivities were less than 0.1 ng/ml. Glucose, albumin, urea, calcium, magnesium, uric acid, inorganic phosphate and creatinine were measured spectrophotometrically by ready-for-use kits supplied by Biolabo® (Maizy, France). The eGFR was calculated by using the Modification of Diet in Renal Disease (MDRD) study equation [25] by applying the following formula:

$$eGFR = 175 \times (S.Cr)^{-1.154} \times (Age)^{-0.203} \times 0.742 \text{ [if female]} \times 1.212 \text{ [if Black]}$$

Ionized calcium (I.Ca) was assessed utilizing the following formula:

$$I.Ca^{2+} = 0.813 \times T.Ca^{0.5} - 0.006 \times Albumin^{0.75} + 0.079 \text{ [26].}$$

Corrected calcium was determined via the following formula:

$$\text{Corrected Ca (mg/dl)} = T.Ca + 0.8 \text{ [4-Albumin].}$$

The total calcium phrase used throughout the study represents the corrected calcium. Serum ionized Mg levels were calculated according to the following formula:

$$I.Mg \text{ (in mM)} = (0.66 \times (T.Mg \text{ in mM})) + 0.039 \quad [27].$$

Statistical Analysis

The Kolmogorov-Smirnov test was used to examine the normality of distribution. Analysis of variance (ANOVA) was applied to examine the between-group differences in scale variables. The analysis evaluated statistical associations between categorical variables by contingency tables (χ^2-test). Pearson's correlation coefficients (r) or Spearman's correlation coefficients (ρ, rho) were calculated to assess correlations between biomarkers. We employed multivariate generalized linear model (GLM) analysis to check the relationship between the biomarkers and the diagnosis (ESRD versus controls) while controlling for background variables including age, BMI, height, weight, family history, tobacco use disorder (TUD) and sex. All statistical analyses were performed using SPSS Statistics version 25 (2017), IBM-USA.

RESULTS

Demographic data and Clinical data

The results of Table 1 showed a significant reduction in the weight and BMI of ESRD patients compared with the control group. At the same time, other parameters showed no significant difference between patients and the control group.

Table 1. Demographic data in ESRD patients and healthy controls

Variables	Control n=33	ESRD n=57	F/Ψ/χ^2	df	p
Age, Yrs.	46.910 ±6.807	46.260 ±10.305	0.103	1/88	0.749
Sex, F/M	11/22	29/28	2.605	1	0.127
Height, cm	161.940 ±11.093	164.950 ±12.334	1.336	1/88	0.251
Weight, kg	74.670 ±12.975	66.280 ±10.271	11.451	1/88	0.001
BMI, kg/m2	28.865 ±6.420	24.394 ±3.090	19.826	1/88	<0.001
Family history, No/Yes	33/0	51/6	3.722	1	0.082
Duration of Disease, Yrs.	-	3.896 ±1.575	-	-	-
TUD, No/Yes	32/1	55/2	0.015	1	1.000

Yrs – Years, F/M – Female/Male, cm – Centimeter, kg – Kilograms, m^2 – Square meter, BMI – Body Mass Index, TUD – Tobacco use disorder, ESRD – End-stage renal disease

Comparison of biochemicals between groups

Table 2 displays the results of the biochemicals measured in the sera of patients and the control group. The results revealed the expected increase of urea, creatinine, uric acid and phosphate in patients, as compared with the control group. At the same time, we saw a significant decrease in eGFR, albumin and total and ionized calcium in the ESRD patients, as compared to the healthy control group. Albumin, magnesium, ionized Mg, Ionized Ca/Mg and Total Ca/Mg were not significantly different between the two groups. Regarding the research parameters, the results showed a significant increase in the serum level of MMP3 and TIMP1 and a significant decrease in the serum level of VitD in the ESRD patients, as compared with the control group.

Table 2. Biochemical data of ESRD patients and healthy controls

Variables	Control n=33	ESRD n=57	F/χ^2	df	p
Creatinine, mg/dl	0.688 ±0.297	8.781 ±3.769	150.888	1/88	<0.001
Urea, mg/dl	28.480 ±7.455	157.190 ±52.905	192.203	1/88	<0.001
eGFR, ml/min/1.73m²	101.554 ±31.183	7.291 ±3.501	513.865	1/88	<0.001
Pi, mg/dl	5.009 ±0.651	7.288 ±866	172.117	1/88	<0.001
Uric acid, mg/dl	4.7091 ±0.835	5.618 ±1.650	8.139	1/88	0.006
Albumin, g/l	43.063 ±6.857	45.404 ±6.611	2.552	1/88	0.114
Magnesium, mM	0.868 ±0.260	0.879 ±0.214	0.045	1/88	0.832
Ionized Mg, mM	0.612 ±0.172	0.619 ±0.141	0.045	1/88	0.832
Calcium, mM	2.266 ±0.175	2.171 ±0.179	6.021	1/88	0.016
Ionized Ca, mM	1.201 ±0.047	1.171 ±0.048	8.368	1/88	0.005
Total Ca/Mg ratio	2.907 ±1.120	2.643 ±0.810	1.675	1/88	0.199
Ionized, Ca/Mg ratio	2.152 ±0.742	2.001 ±0.523	1.270	1/88	0.263
MMP3, ng/ml	47.693 (27.388-73.387)	68.550 (34.172-120.756)	MWUT	-	0.014
TIMP1, ng/ml	486.977 (212.148-706.257)	701.605 (386.775-1045.673)	MWUT	-	0.002
Vitamin D, ng/ml	10.597 (9.660-11.929)	7.961 (7.173-8.913)	MWUT	-	<0.001

BMI – Body mass index, TUD – Tobacco use disorder, eGFR – Estimated glomerular filtration rate, Pi – Inorganic phosphate, MMP3 – Matrix metalloproteinase-3, TIMP1 – Tissue inhibitors of metalloproteinase-1, MWUT – Mann-Whitney U test, Ca – Calcium

Multivariate GLM results

Table 3 shows the multivariate GLM results of the covariates that affect the results of measured biomarkers and the effect size of each covariate. Sex, age, TUD, family history, height, weight and BMI have no significant effects (p>0.05) on the serum level of the biomarkers. The diagnosis (presence of the disease) is the major factor affecting the results of the measured parameters, with a huge effect size (Partial η^2) of 0.824. The top 6 factors that were highly affected by the presence of disease in subjects were eGFR (Partial η^2=0.804), Creatinine (Partial η^2=0.712), Urea (Partial η^2=0.621), Pi (Partial η^2=0.599), uric acid (Partial η^2=0.179), VitD (Partial η^2=0.188). Other parameters show fewer effect sizes. These were not of significant importance.

Intercorrelation matrix

The intercorrelation matrix of the measured biomarkers (MMP3, TIMP1 and VitD) with all other serum parameters is presented in Table 4. The results showed a significant correlation between MMP3 with creatinine (ρ=0.257, p<0.05), urea (ρ = 0.269, p<0.05) and Pi (ρ=0.241, p<0.05). Moreover, a significant inverse correlation was seen between MMP3 and Vit D (ρ=-0.246, p<0.05) and eGFR (ρ=-0.219, p<0.05).

TIMP1 has no significant correlation with other parameters. Serum VitD shows a significant correlation with serum calcium (ρ=0.220, p<0.05), ionized calcium (ρ=0.228, p<0.05), and eGFR (ρ=0.503, p<0.01). There is also a significant negative correlation between Vit D and creatinine (ρ=-0.511, p<0.01), urea (ρ=-0.480, p<0.01), Pi (ρ=-0.545, p<0.01), Uric acid (ρ=-0.238, p<0.05) and MMP3 (ρ=0.246, p<0.01).

Table 3. Multivariate generalized linear model to estimate the effect of the covariates on the levels of the measured biomarker. Test for between subjects was performed to estimate the effect of ESRD on each individual biomarker

Tests	Dependent variables	Independent variables	F	P	Partial η^2
Multivariate	All biomarkers	Diagnosis	46.828	<0.001	0.824
		Sex	0.422	0.885	0.041
		Age	0.651	0.712	0.061
		TUD	0.800	0.590	0.074
		Family history	0.493	0.837	0.047
		Height	0.356	0.925	0.034
		Weight	0.259	0.968	0.025
		BMI	0.304	0.950	0.029
Between-subject effects	eGFR	Diagnosis	337.137	<0.001	0.804
	Creatinine	Diagnosis	199.968	<0.001	0.712
	Urea	Diagnosis	132.905	<0.001	0.621
	Pi	Diagnosis	120.929	<0.001	0.599
	Uric acid	Diagnosis	17.646	<0.001	0.179
	Vitamin D	Diagnosis	18.729	<0.001	0.188
	MMP3	Diagnosis	8.277	0.005	0.093
	Ionized calcium	Diagnosis	4.952	0.029	0.058
	Albumin	Diagnosis	3.985	0.043	0.053
	Calcium	Diagnosis	3.627	0.060	0.043
	T.Ca/Mg	Diagnosis	3.546	0.063	0.042
	Ionized Ca/Mg	Diagnosis	3.072	0.083	0.037
	TIMP1	Diagnosis	1.362	0.247	0.017
	Magnesium	Diagnosis	0.591	0.444	0.007
	Ionized Mg	Diagnosis	0.591	0.444	0.007

BMI – Body mass index, Ca – Calcium, Mg – magnesium, TUD – Tobacco use disorder, eGFR – estimated glomerular filtration rate, Pi – inorganic phosphate, MMP3 – matrix metalloproteinase-3, TIMP1 – tissue inhibitors of metalloproteinase-1

Table 4. Correlation of MMP3, Vitamin D and TIMP1 with other serum biochemicals in ESRD patients

Parameter	MMP3	TIMP1	Vitamin D
Creatinine	0.257*	-0.015	-0.511**
Urea	0.269*	0.067	-0.480**
Pi	0.241*	0.068	-0.545**
Uric acid	0.194	0.111	-0.238*
MMP3	1.000	0.100	-0.246*
TIMP1	0.100	1.000	-0.063
Vitamin D	-0.246*	-0.063	1.000
Albumin	0.176	0.017	-0.021
Magnesium	0.053	-0.006	-0.035
Ionized Mg	0.053	-0.006	-0.035
T.Calcium	-0.059	-0.025	0.220*
Ionized Ca	-0.104	-0.021	0.228*
T.Ca/Mg	-0.076	-0.010	0.113
Ionized Ca/Mg	-0.069	-0.002	0.081
eGFR	-0.219*	0.033	0.503**

* – Significant correlation (p<0.05), ** – Significant correlation (p<0.01)

BMI – Body mass index, TUD – Tobacco use disorder, eGFR – estimated glomerular filtration rate, Pi – inorganic phosphate, MMP3 – matrix metalloproteinase-3, TIMP1 – tissue inhibitors of metalloproteinase-1

DISCUSSION

The major finding of the present study is the significant increase in MMP3 and TIMP1 in ESRD patients compared with the control group, as seen in Table 2. These results are comparable to a recent study that found an increase in several MMPs, including MMP3 and TIMP1, in ESRD patients both before and after hemodialysis, as compared to control values [28]. After dialysis, MMP3 decreased on average in these patients [28]. According to [29], serum MMP3 may be a useful predictor of chronic inflammation [29]. In related work, the median values of TIMP1 were significantly elevated in all dialyzed patients versus controls [30]. MMP3 is involved in the pathogenesis of CKD and is increased in hemodialyzed patients pathogenesis [31]. Since one previous study has shown a tight relationship between the MMP/TIMP system and oxidative stress and inflammation in hemodialysis patients, many possible dialysis-related trigger variables might be a source of such disorders [32]. The proteolytic activity of MMPs is regulated at transcriptional and posttranslational levels and the tissue level by endogenous inhibitors, especially TIMP1 [33].

The changes that occurred in the MMP3 enzyme and its inhibitor (TIMP1) can be explained by the process of inhibition process by the inhibitor. MMP3 in patients with renal failure is a causative agent for inflammation that is usually present in HD patients [32]. The lack of change in the TIMP is due to other mechanisms of the enzyme inhibition process that need more studies to bring to light the exact mechanism for these changes in AKI patients.

The multiple regression analysis in Table 3 assessed the cofounders' effect on all observable parameters. The most significant factor that influenced biomarkers (ESRD diagnosis) was utilized as an explanatory factor to determine the effect of each biomarker after correcting for other cofounders using between-subjects analysis to estimate the effect size of each biomarker by the diagnosis. This was done to investigate the link between biomarkers and ESRD (presence of ESRD in a subject). These tests eliminate cofounders' influence on biomarker results before undertaking association analysis. The results showed that the diagnosis (presence of ESRD in a subject) is the only cofounder that significantly affects the level of the measured biomarkers with a high effect size (Partial η^2=0. 0.824). Therefore, we used the diagnosis only as an explanatory factor to explain its effect on the measured biomarkers.

The routinely measured parameters are increased in ESRD (as expected in this disease). Chronic kidney disease is frequently accompanied by hyperphosphatemia [34]. In treatment, diet, dialysis and drugs are employed simultaneously in an integrated strategy to regulate phosphorus and the other three important CKD mineral bone density laboratory values (calcium, phosphorus and PTH) [35]. In ESRD patients, calcium and phosphorous homeostasis are dysregulated because the kidney plays a vital role in the regulation of these minerals, as well as in active VitD synthesis [36]. Serum urea levels can easily reach or exceed 10 times the upper normal limit, especially when kidney failure occurs. Until recently, urea was considered to be a biologically inert marker. However, urea is a direct and indirect uremic toxin

that is not a reliable assessment of renal function as serum urea concentrations are affected by the hydration state of the patient, dietary intake of protein and liver function [12].

GFR is the most widely accepted standard for assessing renal function in healthy and diseased cases, and estimation of GFR is performed by measuring urea, creatinine or inulin. High serum urea levels can lead to the production and absorption of byproducts which induce the malnutrition and inflammation associated with uremic toxicity [37]. It is suggested that urea predicts cardiovascular disease outcomes beyond other risk factors, including eGFR [38]. The presence of CKD brings about several changes in the renal handling of uric acid, among others, reduced glomerular filtration, enhanced reabsorption, and/or insufficient secretion by renal tubules [39]. Thus, CKD and hyperuricemia often coexist, and serum urate levels increase linearly with decreasing GFR [40].

Kidney failure is characterized by elevated serum phosphorus levels and decreased serum calcium levels due to decreased renal phosphorus excretion (with corresponding increases in serum phosphorus levels) and decreased renal synthesis of active VitD (with corresponding decreases in VitD-mediated calcium uptake from the intestine) [41]. Eventually, the kidney can no longer excrete sufficient phosphorus to maintain homeostasis, resulting in hyperphosphatemia. VitD levels become clinically insufficient as renal function deteriorates and renal phosphorus excretion becomes progressively compromised [42]. Kidney diseases may also lead to magnesium deficiency and, as such, increase the risk of cardiovascular disease [43]. Longstanding CKD is associated with several metabolic disturbances that increase PTH secretion, including hyperphosphatemia, VitD deficiency and hypocalcemia [44].

The intercorrelation study showed that MMP3 is positively correlated with the routinely measured parameters in ESRD, namely urea, creatinine and Pi, and negatively with eGFR. Previous work showed that MMP3 levels could be related to kidney function expressed by eGFR [45]. In related studies, significant positive correlations were observed between MMP3 and both IL-6 and CRP, indicating the mutual effect of the inflammation on the MMP3 level [29]. These results connected MMP3 with the anatomical changes in the kidney tissues in ESRD. TIMP1 may have a role in the pathogenesis of acute renal damage, according to another finding [46]. Furthermore, matrix remodeling occurs in the pathogenesis of CKD, especially at higher baseline levels of total TIMP1 [47,48].

Several studies have revealed that TIMP1 participates in kidney injury by regulating extracellular matrix synthesis and degradation, promoting tubulointerstitial fibrosis through the inhibition of proteolytic matrix metalloproteinases and exacerbating inflammation and renal scarring [49]. In ESRD patients on dialysis, the uremic milieu and the dialysis procedure appear to activate inflammatory cells, creating a pro-inflammatory milieu [50].

CONCLUSION

We introduce another piece of evidence about the alteration in the MMP3 and TIMP1 in ESRD. These biomarkers may act as drug targets for future treatment of ESRD. Our work also demonstrates that ESRD patients suffer from hypovitaminosis D and hypocalcemia, and this needs intervention.

LIMITATIONS OF THE STUDY

The first limitation of the study is the small sample size. We were compelled to utilize a limited study population size to reduce the cost of the study due to the lack of funding. It is recommended to undertake a follow-up study to add more information to the present case-control study.

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