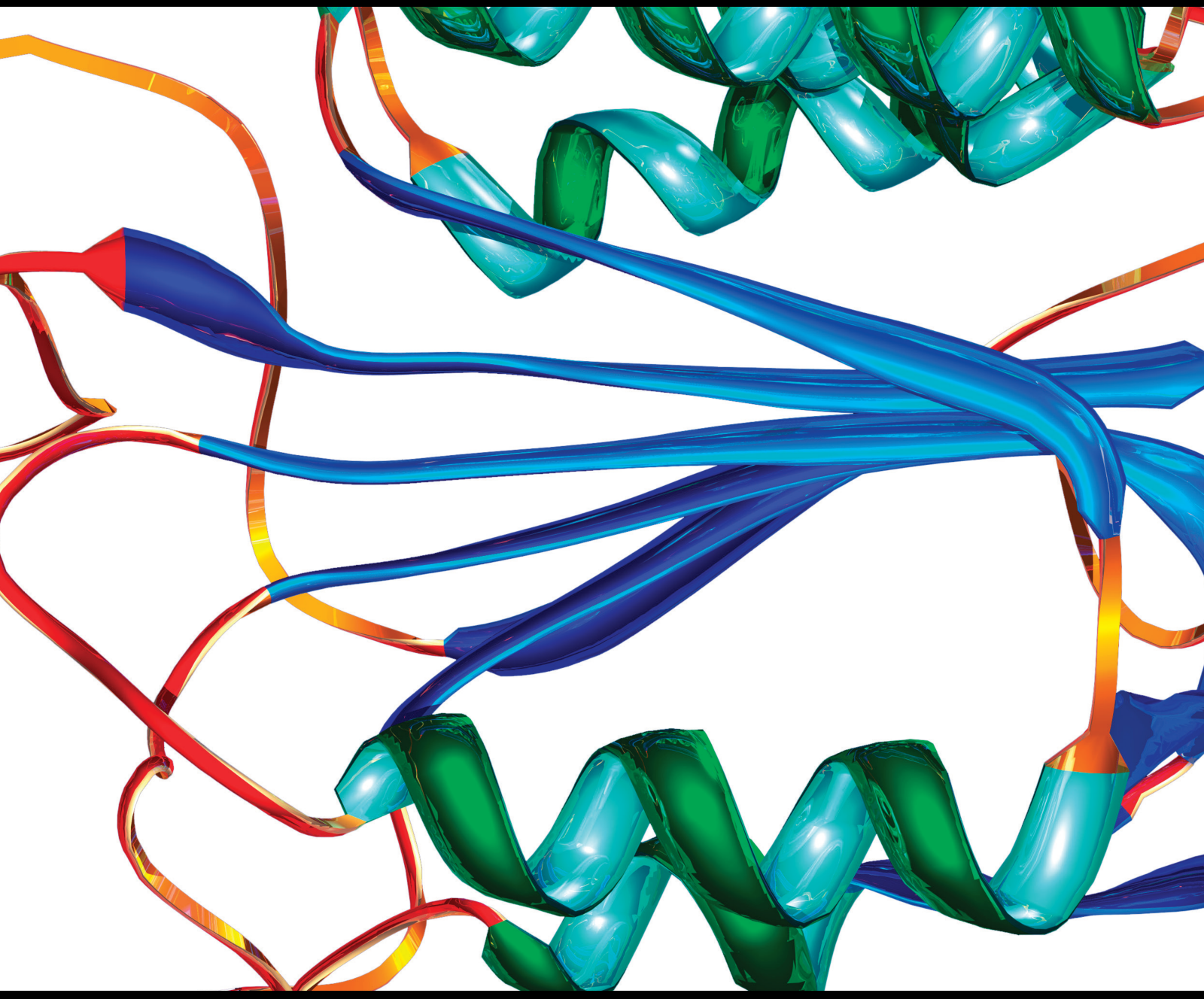
Disease Markers

Novel Biomarkers of Renal Disease Progression

Lead Guest Editor: Carlo Alfieri
Guest Editors: Yuri Battaglia, Dominique Guerrot, Chia-Ter Chao, and Christos Chadjichristos



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Research Article

Growth Differentiation Factor 15 in Children with Chronic Kidney Disease and after Renal Transplantation

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Guest Editor: Christos Chadjichristos

Growth differentiation factor 15 (GDF-15) is strongly associated with cardiovascular disease (CVD). The aim of our study was to evaluate plasma and urinary levels of GDF-15 after pediatric renal transplantation (Rtx) and in children with chronic kidney disease (CKD) and its associations to cardiovascular risk factors. In this cross-sectional study, GDF-15 was measured in plasma and urine from 53 children with a renal transplant and 83 children with CKD and related to cardiovascular risk factors (hypertension, obesity, and cholesterol) and kidney function. Forty healthy children served as a control group. Plasma levels of GDF-15 (median and range) for a Tx (transplantation) cohort, CKD cohort, and healthy controls were, respectively, 865 ng/L (463-3039 ng/L), 508 ng/L (183-3279 ng/L), and 390 ng/L (306-657 ng/L). The CKD and Tx cohorts both had significantly higher GDF-15 levels than the control group ($p < 0.001$). Univariate associations between GDF-15 and hyperuricemia ($p < 0.001$), elevated triglycerides ($p = 0.028$), low HDL ($p = 0.038$), and obesity ($p = 0.028$) were found. However, mGFR ($p < 0.001$) and hemoglobin ($p < 0.001$) were the only significant predictors of GDF-15 in an adjusted analysis. Urinary GDF-15/creatinine ratios were 448 ng/mmol (74–5013 ng/mmol) and 540 ng/mmol (5–14960 ng/mmol) in the Tx cohort and CKD cohort, respectively. In the CKD cohort, it was weakly correlated to mGFR ($r = -0.343$, $p = 0.002$). Plasma levels of GDF-15 are elevated in children with CKD and after Rtx. The levels were not associated with traditional cardiovascular risk factors but strongly associated with renal function.

1. Introduction

Growth differentiation factor 15 (GDF-15), also known as macrophage inhibitory cytokine-1 (MIC-1), is a distant member of the transforming growth factor-β (TGF-β) superfamily. It was originally identified by Bootcov et al. in 1997 as one of the macrophages' regulating factors [1]. The placenta is the only tissue that expresses large amounts of the protein under physiological conditions [2], but its expression is upregulated in various pathological conditions. Elevated levels of GDF-15 are strongly associated with cardiovascular disease (CVD) [3], and in large cohorts, GDF-15 has been shown to be an independent predictor of all-cause mortality when adjusted for cardiovascular risk factors, CVD, and

other biomarkers [4, 5]. GDF-15 seems to have both protective and adverse effects depending on the state of the cells and the microenvironment [6].

To our knowledge, only one study is published on GDF-15 after renal transplantation (Rtx) in adults [7]. In that study, GDF-15 was related to anemia and hepcidin, indicating its involvement in the pathogenesis of anemia. In addition, GDF-15 was related to creatinine and estimated glomerular filtration rate (eGFR). Urinary GDF-15 levels have also been shown to be elevated and negatively correlated with eGFR in adults with diabetes [8]. Increasing data exists on GDF-15 in children, but only one study on children with kidney disease is published and demonstrates elevated GDF-15 levels in patients on hemodialysis and peritoneal dialysis [9].

We hypothesized that circulating GDF-15 is associated with cardiovascular risk factors in children after Rtx and that plasma and urinary GDF-15 could be used as a biomarker of CVD risk in children. We also wanted to adjust the relation between GDF-15 and CVD risk factors for kidney function as adult studies have indicated a relation between kidney function and GDF-15.

2. Material and Methods

2.1. Patient Cohorts. Tx cohort: children and adolescents ≤ 18 years of age who underwent Rtx at Oslo University Hospital between 2000 and 2015. The patients participated in the HENT (Health after Kidney Transplantation) study and patients were enrolled in 2015-16. Inclusion criteria for the HENT study were a functioning graft for at least 1 year and no ongoing signs of rejection.

CKD cohort: children and adolescents < 18 years of age with CKD were included in a cross-sectional study, evaluating biomarkers in CKD and different methods of measuring glomerular filtration rate (mGFR). The children were in a stable phase of their CKD and enrolled at the pediatric departments at Oslo University Hospital and Haukeland University Hospital [10, 11].

Written informed consent was obtained from patients and/or their parents prior to start of the study. The study protocols were approved by the Regional Committee for Medical and Health Research Ethics (references 2009/1008 and 2009/741), and the study was carried out according to the Declaration of Helsinki.

2.2. Healthy Control Group. Blood samples from a healthy group of fasting children aged 5-8 years were used as the control group for circulating GDF-15 levels. These healthy children, without any sign of CVD or renal disease, were included as part of a longitudinal pregnancy follow-up study of mother and children after pregnancy complications, i.e., preeclampsia and diabetes mellitus (gestational and type 1) [12, 13].

2.3. Anthropometrics. Body Mass Index (BMI) was calculated as kg/m^2. *Z*-scores for weight, height, and BMI were calculated based on the LMS method, using Norwegian references [14], and overweight and obesity was defined according to BMI cut-off limits proposed by the International Obesity Task Force (isoBMI > 25 for overweight and isoBMI > 30 for obesity) [15].

2.4. Renal Function. mGFR was measured by using an injection of Omnipaque® (GE Healthcare, Oslo, Norway; i.e., 647 mg iohexol/mL) with blood sampling after 2 and 5 hours as described in a previous publication [10]. In the Tx cohort, 2 mL of Omnipaque® was given to children under 2 years and 5 mL to children over 2 years while the dose was adjusted to the child's weight in the CKD cohort (<10 kg, 1 mL; 10–20 kg, 2 mL; 20–30 kg, 3 mL; 30-40 kg, 4 mL; and >40 kg, 5 mL).

2.5. Blood Pressure. Blood pressure was measured using automatic blood pressure monitors. Hypertension was defined as systolic blood pressure (SBP) or diastolic blood pressure (DBP) over the 95th percentile for age, height, and gender and/or use of antihypertensive medication [16].

2.6. Biochemistry. Venous blood samples were obtained after an overnight fast. Hemoglobin was measured by photometry (Sysmex XN). Plasma HDL cholesterol, LDL cholesterol, total cholesterol, and uric acid were measured by enzymatic colorimetric methods and plasma triglycerides by an enzymatic photometric method (Cobas® c702, Roche Diagnostics). The following thresholds were used as definition for cardiovascular risk factors: P-HDL < 40 mg/dL (1.03 mmol/L), P-LDL > 130 mg/dL (3.36 mmol/L), P-cholesterol > 200 mg/dL (5.17 mmol/L), and P-triglycerides > 150 mg/dL (1.7 mmol/L). Uric acid levels were adjusted with age- and gender-specific normal values, and the 95th percentile was used as the cut-off value for the definition of hyperuricemia [17].

2.7. GDF-15. In the two study cohorts, plasma GDF-15 was measured in duplicate, after one freeze-thaw cycle (two cycles for the CKD cohort), by a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) with a human GDF-15 Quantikine® ELISA kit (Bio-Techne). Urinary GDF-15 was measured in duplicate by the same GDF-15 Quantikine® ELISA kit and normalized for urine creatinine.

In the control group, GDF-15 was measured in plasma by an immunoradiometric sandwich assay using a polyclonal, affinity chromatography-purified goat antihuman GDF-15 IgG antibody (R&D Systems). The analyses were performed in duplicate at the laboratory where the assay was developed.

According to a recent study [18], there is a good correlation between the two different methods of measuring GDF-15 in plasma.

2.8. Statistical Analysis. Data are described as either median and range or geometric means with 95% confidence interval. Natural logarithmic transformations were performed for achieving more normally distributed data due to a positively skewed distribution of plasma GDF-15 levels and urinary GDF-15/creatinine ratio. For two continuous variables, the strength of associations was measured using Pearson or Spearman correlations depending on the distribution of the data. For continuous variables, the difference between two groups was analyzed using a Mann-Whitney Wilcoxon test or a *t*-test depending on the distribution of the data.

Table 1: Basal characteristics of the two study cohorts and the control group. Values in median and range.

	Tx cohort	CKD cohort	Healthy controls
N	53	83	40
Age (years)	12.2 (2.3–18.0)	10.1 (2.0–17.5)	6.7 (4.8–8)
Male (*n*, %)	32 (60%)	49 (59%)	
Weight (kg)	39.3 (11.1–90.4)	30.8 (8.96–84.6)	
Weight *Z*-score	-0.45 (-2.60–3.10)	-0.31 (-3.43–2.66)	
Height (cm)	142 (83–184)	137 (74–177)	
Height *Z*-score	-1.52 (-4.4–0.5)	-0.53 (-4.63–2.04)	
BMI (kg/m^2)	17.9 (14.2–35.4)	17.0 (12.7–33.2)	
BMI *Z*-score	0.34 (-1.49–2.97)	0.20 (-3.30–2.75)	
Overweight/obesity (*n*, %)	12/5 (23/9)	11/3 (13/4)	
Age at Rtx1 (years)	4.4 (0.8–15.8)	—	
Time from Rtx1 (years)	5.0 (1.0–15.5)	—	
Preemptive Rtx1 (*n*, %)	25 (47%)	—	
Total dialysis (months)	9.5 (0.25–39.5)	—	
Rtx1/Rtx2	51/2	—	
LD/DD (*n*, %)	48/5 (91%)	—	
mGFR (mL/min/1.73 m^2)[a]	56 (24–111)	73 (14–143)	
Hemoglobin (g/dL)	12.2 (7.1–14.8)	12.5 (8.7–15.5)	
HbA1c (%)	5.2 (4.2–7.8)	—	
Protein/creatinine ratio (mg/mmol)	16 (6–193)	27 (3–1084)	
<15 mg/mmol (*n*, %)	24 (46%)	26 (31%)	
15–50 mg/mmol (*n*, %)	22 (42%)	29 (35%)	
>50 mg/mmol (*n*, %)	6 (11%)	28 (34%)	
Etiology of ESRD/CKD			
CAKUT	23 (43%)	27 (33%)	
Hereditary	13 (25%)	23 (28%)	
Glomerulonephritis	8 (15%)	9 (11%)	
Acquired	7 (13%)	10 (12%)	
Vesiculoureter reflux	—	7 (8%)	
Miscellaneous/unknown	2 (4%)	7 (8%)	

[a]For two patients in the Tx cohort, the mGFR is missing because of low GFR, replaced with eGFR.

Multivariate linear regression was chosen for adjusted analysis of associations between LnGDF-15 and potential explanatory variables. All statistical analyses were performed in Statistical Package for Social Sciences (SPSS) version 21.

3. Results

3.1. Patient Characteristics. Fifty-three children (32 boys, median age 12.2 years, range 2.3–18 years) with a renal transplant were included. The causes of ESRD were congenital anomalies of the kidney and urinary tract (CAKUT) ($n = 23$), hereditary causes ($n = 13$), glomerulonephritis ($n = 8$), acquired (excluding glomerulonephritis) ($n = 7$), and other or unknown etiologies ($n = 2$). The individual GFR measurements were distributed according to different CKD stages in the following way: 5, 17, 30, and 1 patients in CKD stages 1, 2, 3 and 4, respectively. Eighty-three children with CKD (49 boys, median age 10.1 years, range 2.0-17.5 years) were enrolled, 34 from Oslo University Hospital and 49 from Haukeland University Hospital. The distribution according to CKD stages was as follows: 27, 24, 19, and 13 patients in CKD stages 1, 2, 3, and 4–5, respectively. 11% of the Tx patients and 34% of the CKD patients had significant proteinuria (protein/creatinine ratio > 50 mg/mmol). The patients' basal characteristics and demographics are presented in Table 1.

3.2. Immunosuppression. The majority of patients in the Tx group received a tacrolimus-based immunosuppression ($n = 47$), combined with mycophenolate ($n = 29$) and prednisolone ($n = 48$, mean daily dose 0.071 mg/kg). CsA was used in seven patients and nine received everolimus (three as a monotherapy with prednisolone, five in combination with a calcineurin inhibitor, and one with mycophenolate). Azathioprine was used by three patients (in combination with a calcineurin inhibitor and prednisolone). In the CKD group, five patients (6%) received immunosuppressive treatment, one tacrolimus and mycophenolate because of previous limbal transplantation and the remaining four received tacrolimus, mycophenolate, and/or prednisolone

Table 2: Plasma and urinary levels of GDF-15 in the two study cohorts (median and range).

	Tx cohort	CKD cohort	Healthy controls
Plasma GDF-15 (ng/L)	865 (463–3039)	508 (183–3279)	390 (306–657)
Urinary GDF-15 (ng/L)	2740 (449–9183)	2263 (41–28760)	NA
Urinary GDF-15/creatinine ratio (ng/mmol)	448 (74–5013)	540 (5–14960)	NA

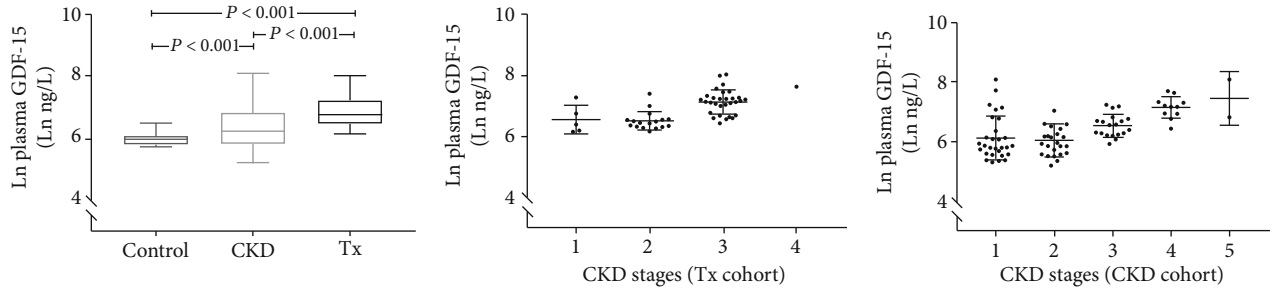

Figure 1: Comparison of plasma GDF-15 levels (mean ± SD) in the Tx cohort, CKD cohort, and healthy controls. Distribution of plasma GDF-15 values (mean ± SD) according to CKD stages in the Tx cohort (b) and CKD cohort (c). Shown in natural logarithmic (Ln) transformation due to skewed distribution.

Table 3: Prevalence of cardiovascular risk factors in the Tx cohort and univariate relations to GDF-15.

		N (%)	Geometric mean (ng/L)	95% CI	*p* value
Weight	Normal weight	36 (68%)	937	796-1103	0.028* ANOVA
	Overweight	12 (23%)	857	639-1150	
	Obesity	5 (9%)	1647	1288-2107	
Blood pressure	Hypertension	27 (49%)	967	799-1170	0.981
	No hypertension	26 (51%)	970	794-1186	
HDL	<40 mg/dL	11 (21%)	1277	889-1832	0.038*
	≥40 mg/dL	41 (79%)	907	786-1047	
LDL	>130 mg/dL	5 (9%)	1103	667-1825	0.536
	≤130 mg/dL	48 (91%)	956	828-1103	
Cholesterol	>200 mg/dL	9 (17%)	952	821-1104	0.462
	≤200 mg/dL	43 (83%)	1088	718-1646	
TG	>150 mg/dL	33 (62%)	1085	914-1288	0.028*
	≤150 mg/dL	20 (38%)	803	655-986	
Uric acid	Hyperuricemia	22 (42%)	1288	668-938	<0.001*
	No hyperuricemia	31 (58%)	792	1096-1512	

*Two-sided *p* value less than 0.05.

as a treatment for glomerular diseases (two glomerulonephritides, steroid-resistant nephrotic syndrome, and Henoch-Schonlein purpura).

3.3. Plasma GDF-15 Levels in Children with Renal Failure. The respective plasma GDF-15 levels (median and range) for the Tx cohort, CKD cohort, and the control group were 865 ng/L (463-3039 ng/L), 508 ng/L (183-3279 ng/L), and 390 ng/L (306-657 ng/L) (Table 2). As shown in Figure 1, the Tx cohort had significantly higher plasma GDF-15 levels than both the CKD cohort ($p < 0.001$) and the control group ($p < 0.001$). Figure 1 shows as well the distribution of plasma GDF-15 according to the different CKD stages. Plasma GDF-15 levels were also significantly higher in the CKD cohort than the control group ($p < 0.001$). There were no significant differences in plasma GDF-15 levels between genders in either study group.

3.4. Plasma GDF-15 Levels and Cardiovascular Risk Factors. 23% and 9% of the patients in the Tx cohort had overweight

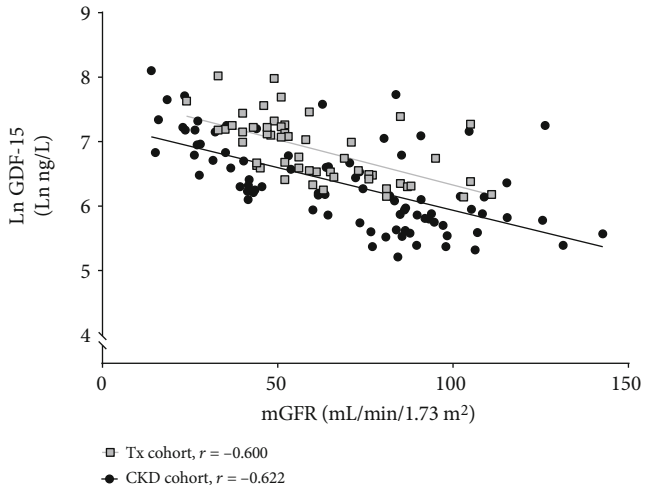

Figure 2: Univariate correlations between plasma GDF-15 and mGFR in Tx and CKD cohorts.

and obesity, respectively. 51% had hypertension and up to 62% had some kind of dyslipidemia (Table 3). In univariate analyses, plasma levels of GDF-15 were significantly higher in obese patients ($p = 0.028$), in patients with high levels of triglycerides ($p = 0.028$), and in patients with low levels of HDL cholesterol ($p = 0.038$). 42% had hyperuricemia and those had significantly higher plasma levels ($p < 0.001$), and uric acid was significantly correlated with plasma GDF-15 ($r = 0.451$, $p = 0.001$) and mGFR ($r = -0.604$, $p < 0.001$). For the other cardiovascular risk factors, there were no significant differences in GDF-15 levels. Only one patient had diabetes mellitus type 1 with a slightly elevated HbA1c (7.8%). Hemolytic uremic syndrome (HUS) was the cause of ESRD in this patient, and the patient developed diabetes as a result of pancreas infarcts during the initial presentation of HUS. The rest of the patients in the Tx cohort had normal HbA1c (Table 1).

3.5. Plasma GDF-15 Levels and Renal Function. Plasma GDF-15 levels had a significant negative correlation with mGFR in both the Tx cohort ($r = -0.600$, $p < 0.001$) and the CKD cohort ($r = -0.622$, $p < 0.001$). The distribution is similar in both groups as shown in Figure 2, and the correlation was also significant when the two groups are merged ($r = -0.616$, $p < 0.001$). There was no statistically significant difference in mGFR between the Tx cohort and the CKD cohort ($p = 0.140$). Hemoglobin was negatively correlated to plasma GDF-15 levels in both groups and for the two groups combined ($r = -0.580$, $p < 0.001$). There were no significant correlations between plasma GDF-15 levels and age in either the two groups separately or the combined group.

In a multivariate model where the two groups were merged for gaining statistical power, mGFR, hemoglobin, and the study group were significant predictors of plasma GDF-15 (Table 4). In a subanalysis for the Tx cohort where cardiovascular risk factors (hypertension, triglycerides, and cholesterol) were taken in as possible explanatory factors in addition to mGFR, age, and sex, mGFR was the only significant predictor ($p < 0.001$). Due to multicollinearity, uric acid was not included in the multivariate analysis.

3.6. Urinary GDF-15. There was not a significant difference in urinary GDF-15/creatinine ratio between the Tx cohort and the CKD cohort (Table 2). No significant associations were found with cardiovascular risk factors in either group. In the CKD cohort, there was a significant correlation between urinary GDF-15/creatinine ratio and mGFR ($r = -0.343$, $p = 0.002$). In the Tx cohort, urine was only available from 50/53 patients and there was no significant correlation between the GDF-15/creatinine ratio and mGFR in this group ($r = 0.077$, $p = 0.597$). The urinary GDF-15/creatinine ratio correlated positively with plasma GDF-15 levels in the Tx cohort ($r = 0.408$, $p = 0.003$) and the CKD cohort ($r = 0.422$, $p < 0.001$).

4. Discussion

In this study, we found that plasma levels of GDF-15 are significantly elevated in children with a renal transplant and in children with chronic kidney diseases compared to healthy children and that plasma GDF-15 levels are strongly associated with kidney function.

To our knowledge, this is the first time GDF-15 has been related to kidney function in a pediatric cohort although an association between renal function and plasma GDF-15 has been found in adults [7, 19]. The knowledge of associations between GDF-15 and renal disease has been increasing. GDF-15 has been suggested as an independent risk factor of mortality in adults with end stage renal disease (ESRD) [19, 20] and for progression of kidney disease [21]. Elevated circulating GDF-15 has been related to incident kidney disease, and it is suggested that it might be useful in predicting the progression of chronic kidney disease, years before clinical onset of the disease [22]. Studies in healthy males and in adults with diabetic nephropathy have shown a faster decline of GFR in patients with high levels of GDF-15 [19, 23]. The role of GDF-15 in decreasing renal function is poorly understood, but in murine models, GDF-15 plays a significant role in the proliferation of acid-secreting intercalated cells in the collecting duct [24] and is an early mediator after induced kidney injury [25].

GDF-15 is a member of the TGF-β superfamily, and TGF-β is a mediator of fibrosis and inflammation [26]. TGF-β is an important profibrotic factor in the kidneys and plays a role in endothelial-to-mesenchymal transition that is suggested to be important in chronic allograft tubular atrophy/interstitial fibrosis [27]. GDF-15 has also been associated with fibrosis in diseases of other organ systems such as dilated cardiomyopathy [28], systemic sclerosis [29], and chronic liver disease [30]. Two recent studies have shown GDF-15 to be associated with biopsy-proven fibrosis in the kidneys, the first in patients with IgA nephropathy [31] and the other in idiopathic membranous nephropathy [32]. GDF-15 might therefore also be a marker or a causative factor of kidney fibrosis that is responsible for decreased renal function in our pediatric cohorts.

Nair et al. found a significant correlation between intrarenal tubulointerstitial expression of GDF-15 mRNA and circulating GDF-15 in 24 patients with CKD [21] which implies that it is produced in the kidneys and might have a

Table 4: Multiple linear regression model for plasma GDF-15 (Tx and CKD cohorts).

Dependent variable GDF-15 Risk factor	Unstandardized B	p value	95% CI for B
Age (years)	0.005	0.613	(-0.015, 0.026)
Sex	0.085	0.286	(-0.072, 0.243)
mGFR (mL/min/1.73 m^2)	-0.009	<0.001	(-0.012, -0.006)
Hemoglobin (g/dL)	-0.162	<0.001	(-0.219, -0.115)
Hypertension	0.004	0.956	(-0.152, 0.161)
BMI Z-score	-0.096	0.445	(-0.344, 0.152)
Height Z-score	-0.146	0.120	(-0.330, 0.036)
Weight Z-score	0.113	0.454	(-0.185, 0.411)
CKD vs. Tx	0.262	0.005	(0.008, 0.445)

pathophysiological role in the progression of CKD and/or the development of interstitial fibrosis. If it is excreted in urine, it could be a valuable, noninvasive marker of kidney function or kidney fibrosis. We found, however, only a weak correlation between urinary GDF-15 and renal function in our CKD cohort and no significant correlation in the Tx cohort. We therefore cannot postulate urinary GDF-15 as a biomarker of either renal function or renal fibrosis. Due to the correlation between GDF-15 and renal function in the present study and strong associations between circulating GDF-15 and fibrosis in other organ systems, we consider the relationship between GDF-15 (urinary and circulating) and renal fibrosis to be worth further exploration.

Serum GDF-15 levels are elevated in disorders of ineffective erythropoiesis such as thalassemia [33], and GDF-15 is a possible mediator of anemia through hepcidin in adult renal transplant recipients [7]. Hepcidin plays an important role in iron metabolism as it negatively regulates plasma iron levels by binding to ferroportin which induces internalization of iron into the reticuloendothelial system. Hepcidin levels are elevated in kidney failure due to decreased renal clearance and inflammatory upregulation which results in reduced availability of plasma iron and anemia [34]. We found strong correlations between plasma GDF-15 and hemoglobin that supports the relationship of GDF-15 to erythropoiesis, but hepcidin levels were not measured in our patients. Hemoglobin and mGFR are interrelated in CKD and our study revealed hemoglobin and mGFR to be equally strong predictors of plasma GDF-15, but this cross-sectional study does not allow us to determine the causal factor in this relationship.

In the adult population, plasma GDF-15 has been associated with progression and prognosis of CVD [3] and may be a potential tool for risk stratification of CVD [35]. We therefore hypothesized that it would be associated to cardiovascular risk factors in our Tx cohort. The prevalence of cardiovascular risk factors in our group of renal transplanted children is high, and we found significant univariate associations between GDF-15, hyperuricemia, elevated triglycerides, low HDL, and obesity. We found, however, that renal function is a major determinant of plasma GDF-15 in children with reduced kidney function. mGFR and hemoglobin were the only significant predictors of GDF-15 in adjusted analysis. Thus, we conclude that while plasma GDF-15 is associated (in unadjusted analyses) with cardiovascular risk factors in renal transplanted children, it is not useful as a biomarker for cardiovascular disease in this group because of the very strong association with renal function.

There are some limitations to our study. Plasma GDF-15 was measured by a different method in the healthy control group. There has however been published a study that compares different methods to measure GDF-15, and it shows a good correlation between the two methods [18]. We are therefore confident that the comparison is reliable. In addition, the study groups are small and heterogeneous with regard to age and underlying diseases. A small sample increases the probability of a type 2 error, but when we have a significant finding, this is less relevant. On the other hand is the Tx group representative for the whole Norwegian population as patients were recruited from the whole country with a high participation rate.

In conclusion, circulating GDF-15 levels are elevated in children after kidney transplantation and in children with decreased renal function. While we found significant univariate associations between GDF-15 and risk factors for CVD as elevated triglycerides, low HDL and obesity, mGFR, and hemoglobin were the only significant predictors of GDF-15 in an adjusted analysis. We found that GDF-15 is associated with renal function in children, and this strong association does not make plasma GDF-15 a useful biomarker for CVD in this population. Whether GDF-15 might be useful in evaluation of kidney fibrosis should be evaluated further. Evaluation of fibrosis in transplant biopsies and possible associations with circulating GDF-15 could be a field of future research at centers where routine surveillance biopsies are performed.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] M. R. Bootcov, A. R. Bauskin, S. M. Valenzuela et al., "MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily," *Proceedings of the National Academy of Sciences*, vol. 94, no. 21, pp. 11514–11519, 1997.

[2] A. G. Moore, D. A. Brown, W. D. Fairlie et al., "The transforming growth factor-ss superfamily cytokine macrophage inhibitory cytokine-1 is present in high concentrations in the serum of pregnant women," *The Journal of clinical endocrinology and metabolism*, vol. 85, no. 12, pp. 4781–4788, 2000.

[3] K. C. Wollert, T. Kempf, and L. Wallentin, "Growth differentiation factor 15 as a biomarker in cardiovascular disease," *Clinical chemistry*, vol. 63, no. 1, pp. 140–151, 2017.

[4] F. E. Wiklund, A. M. Bennet, P. K. Magnusson et al., "Macrophage inhibitory cytokine-1 (MIC-1/GDF15): a new marker of all-cause mortality," *Aging Cell*, vol. 9, no. 6, pp. 1057–1064, 2010.

[5] T. J. Wang, K. C. Wollert, M. G. Larson et al., "Prognostic utility of novel biomarkers of cardiovascular Stress," *Circulation*, vol. 126, no. 13, pp. 1596–1604, 2012.

[6] J. Corre, B. Hebraud, and P. Bourin, "Concise review: growth differentiation factor 15 in pathology: a clinical role?," *Stem cells translational medicine*, vol. 2, no. 12, pp. 946–952, 2013.

[7] J. Malyszko, E. Koc-Zorawska, J. S. Malyszko, I. Glowinska, M. Mysliwiec, and I. C. Macdougall, "GDF15 is related to anemia and hepcidin in kidney allograft recipients," *Nephron Clinical practice*, vol. 123, no. 1-2, pp. 112–117, 2013.

[8] M. S. Simonson, M. Tiktin, S. M. Debanne et al., "The renal transcriptome of db/db mice identifies putative urinary biomarker proteins in patients with type 2 diabetes: a pilot study," *American journal of physiology Renal physiology*, vol. 302, no. 7, pp. F820–F829, 2012.

[9] A. Bargenda, K. Musial, and D. Zwolinska, "Epidermal growth factor, growth differentiation factor-15, and survivin as novel biocompatibility markers in children on chronic dialysis," *Biomarkers*, vol. 21, no. 8, pp. 752–756, 2016.

[10] C. Tøndel, B. Bolann, C. L. Salvador et al., "Iohexol plasma clearance in children: validation of multiple formulas and two-point sampling times," *Pediatric nephrology*, vol. 32, no. 2, pp. 311–320, 2017.

[11] C. Tøndel, C. L. Salvador, K. O. Hufthammer et al., "Iohexol plasma clearance in children: validation of multiple formulas and single-point sampling times," *Pediatric nephrology*, vol. 33, no. 4, pp. 683–696, 2018.

[12] A. S. Kvehaugen, R. Dechend, H. B. Ramstad, R. Troisi, D. Fugelseth, and A. C. Staff, "Endothelial function and circulating biomarkers are disturbed in women and children after preeclampsia," *Hypertension*, vol. 58, no. 1, pp. 63–69, 2011.

[13] A. S. Kvehaugen, L. F. Andersen, and A. C. Staff, "Anthropometry and cardiovascular risk factors in women and offspring after pregnancies complicated by preeclampsia or diabetes mellitus," *Acta obstetricia et gynecologica Scandinavica*, vol. 89, no. 11, pp. 1478–1485, 2010.

[14] P. B. Júlíusson, M. Roelants, E. Nordal et al., "Growth references for 0-19 year-old Norwegian children for length/height, weight, body mass index and head circumference," *Annals of human biology*, vol. 40, no. 3, pp. 220–227, 2013.

[15] T. J. Cole, M. C. Bellizzi, K. M. Flegal, and W. H. Dietz, "Establishing a standard definition for child overweight and obesity worldwide: international survey," *BMJ*, vol. 320, no. 7244, pp. 1240–1243, 2000.

[16] V. A. Moyer, "Screening for primary hypertension in children and adolescents: U.S. Preventive Services Task Force recommendation statement," *Annals of internal medicine*, vol. 159, no. 9, pp. 613–619, 2013.

[17] M. Kubota, A. Nagai, L. Tang, and M. Tokuda, "Investigation on hyperuricemia in children with obesity or various pediatric disorders," *Nucleosides, nucleotides & nucleic acids*, vol. 30, no. 12, pp. 1051–1059, 2011.

[18] K. C. Wollert, T. Kempf, E. Giannitsis et al., "An automated assay for growth differentiation factor 15," *The Journal of Applied Laboratory Medicine: An AACC Publication*, vol. 1, no. 5, pp. 510–521, 2017.

[19] M. Lajer, A. Jorsal, L. Tarnow, H. H. Parving, and P. Rossing, "Plasma growth differentiation factor-15 independently predicts all-cause and cardiovascular mortality as well as deterioration of kidney function in type 1 diabetic patients with nephropathy," *Diabetes Care*, vol. 33, no. 7, pp. 1567–1572, 2010.

[20] S. N. Breit, J. J. Carrero, V. W. W. Tsai et al., "Macrophage inhibitory cytokine-1 (MIC-1/GDF15) and mortality in end-stage renal disease," *Nephrology Dialysis Transplantation*, vol. 27, no. 1, pp. 70–75, 2012.

[21] V. Nair, C. Robinson-Cohen, M. R. Smith et al., "Growth differentiation factor-15 and risk of CKD progression," *Journal of the American Society of Nephrology*, vol. 28, no. 7, pp. 2233–2240, 2017.

[22] J. E. Ho, S. J. Hwang, K. C. Wollert et al., "Biomarkers of cardiovascular stress and incident chronic kidney disease," *Clinical chemistry*, vol. 59, no. 11, pp. 1613–1620, 2013.

[23] A. C. Carlsson, E. Ingelsson, J. Sundström et al., "Use of proteomics to investigate kidney function decline over 5 years," *Clinical journal of the American Society of Nephrology*, vol. 12, no. 8, pp. 1226–1235, 2017.

[24] J. P. Duong Van Huyen, L. Cheval, M. Bloch-Faure et al., "GDF15 triggers homeostatic proliferation of acid-secreting collecting duct cells," *Journal of the American Society of Nephrology*, vol. 19, no. 10, pp. 1965–1974, 2008.

[25] T. A. Zimmers, X. Jin, E. C. Hsiao, S. A. McGrath, A. F. Esquela, and L. G. Koniaris, "Growth differentiation factor-

15/macrophage inhibitory cytokine-1 induction after kidney and lung injury," *Shock*, vol. 23, no. 6, pp. 543–548, 2005.

[26] E. P. Böttinger, "TGF-beta in renal injury and disease," *Seminars in Nephrology*, vol. 27, no. 3, pp. 309–320, 2007.

[27] S. Bedi, A. Vidyasagar, and A. Djamali, "Epithelial-to-mesenchymal transition and chronic allograft tubulointerstitial fibrosis," *Transplantation reviews*, vol. 22, no. 1, pp. 1–5, 2008.

[28] S. I. Lok, B. Winkens, R. Goldschmeding et al., "Circulating growth differentiation factor-15 correlates with myocardial fibrosis in patients with non-ischaemic dilated cardiomyopathy and decreases rapidly after left ventricular assist device support," *European journal of heart failure*, vol. 14, no. 11, pp. 1249–1256, 2012.

[29] K. Yanaba, Y. Asano, Y. Tada, M. Sugaya, T. Kadono, and S. Sato, "Clinical significance of serum growth differentiation factor-15 levels in systemic sclerosis: association with disease severity," *Modern rheumatology*, vol. 22, no. 5, pp. 668–675, 2012.

[30] M. Krawczyk, S. Zimmermann, G. Hess et al., "Panel of three novel serum markers predicts liver stiffness and fibrosis stages in patients with chronic liver disease," *PLoS One*, vol. 12, no. 3, article e0173506, 2017.

[31] K. R. Na, Y. H. Kim, H. K. Chung et al., "Growth differentiation factor 15 as a predictor of adverse renal outcomes in patients with immunoglobulin A nephropathy," *Internal medicine journal*, vol. 47, no. 12, pp. 1393–1399, 2017.

[32] Y. R. Ham, C. H. Song, H. J. Bae et al., "Growth differentiation factor-15 as a predictor of idiopathic membranous nephropathy progression: a retrospective study," *Disease markers*, vol. 2018, Article ID 1463940, 9 pages, 2018.

[33] T. Tanno, P. Noel, and J. L. Miller, "Growth differentiation factor 15 in erythroid health and disease," *Current opinion in hematology*, vol. 17, no. 3, pp. 184–190, 2010.

[34] W. Hayes, "Measurement of iron status in chronic kidney disease," *Pediatric nephrology*, vol. 34, no. 4, pp. 605–613, 2018.

[35] M. Krintus, M. Kozinski, J. Kubica, and G. Sypniewska, "Critical appraisal of inflammatory markers in cardiovascular risk stratification," *Critical reviews in clinical laboratory sciences*, vol. 51, no. 5, pp. 263–279, 2014.

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Research Article

Urinary Matrix Metalloproteinase-7 and Prediction of AKI Progression Post Cardiac Surgery

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Aims. Early detection of patients at high risk for progressive acute kidney injury (AKI) after cardiac surgery remains a major challenge. We aim to evaluate the utility of urinary matrix metalloproteinase-7 (uMMP-7) and other reported biomarkers for predicting AKI progression during postoperative hospital stay. *Methods*. We conducted a prospective, multicenter cohort study in 121 adult patients with stage 1 or 2 AKI after cardiac surgery. uMMP-7 and other well-reported biomarkers (uIL-18, uNGAL, and UACR) were measured at time of AKI clinical diagnosis. The primary outcome is the progression of AKI after cardiac surgery, defined as worsening of AKI stage (stage 1 to either stage 2 or stage 3 or from stage 2 to stage 3). *Results*. A level of uMMP-7 > 7.8 μg/g Cr at time of AKI diagnosis conveyed an 8-fold risk of AKI progression as compared to those with uMMP-7 < 2.7 μg/g after adjusting for clinical risk factors. The performance of uMMP-7 for predicting progressive AKI was good with an AUC of 0.80. The combination of uMMP-7 and IL-18 produces the greatest AUC for predicting progressive AKI. Addition of uMMP-7 to the clinical risk factor model significantly improved risk reclassification for AKI progression. *Conclusions*. uMMP-7, measured at time of AKI clinical diagnosis, is a novel biomarker for predicting the progression of AKI after cardiac surgery. Adding uMMP-7 to the clinical risk factor model may be used as a noninvasive approach to identify a subpopulation that is at high risk for progressive AKI after cardiac surgery.

1. Introduction

Acute kidney injury (AKI) is common in patients who are receiving cardiac surgery by cardiopulmonary bypass, with high morbidity (ranging 30-40%) [1] and mortality (ranging 10-20%) [2]. The episodes and severity of AKI were linked to all kinds of poor outcomes and increased risk of future chronic kidney disease (CKD) and even end-stage kidney disease [3].

Most patients who develop AKI after cardiac surgery experience a mild form of AKI (e.g., Kidney Disease Improving Global Outcomes (KDIGO) stage 1). However, about 10%–15% of patients with initial mild AKI after surgery will progress to a more severe stage (KDIGO stage 2 or 3) or even require dialysis during their postoperative hospital stay [4, 5]. Recent studies have consistently shown that risk of mortality exponentially increased with increasing stages of AKI [6, 7]. Early identification of patients at high risk for progressive AKI after cardiac surgery would help physicians to improve monitoring and care of postoperative patients, guide patient counseling and decision-making, and facilitate participation in interventional trials of AKI [5].

During the past decade, there has been increasing interest on searching new biomarkers for AKI development and prognosis in the setting of cardiac surgery. Several biomarkers, such as NGAL (neutrophil gelatinase-associated lipocalin) and IL-18 (interleukin-18), were found to predict AKI development before the clinical diagnosis was reached [8]. Furthermore, the biomarker level can also predict in-hospital outcomes, such as receiving renal replacement therapy, long hospital stays, and in-hospital mortality. A few studies also showed that biomarkers measured at the early

stage of established AKI predicted the progression of AKI after cardiac surgery but yielded modest performance in general [4, 5].

Matrix metalloproteinase-7 (MMP-7) is one of the smallest secreted matrix metalloproteinases, predominantly localized in renal tubular epithelium, and can be easily excreted into urine [9]. Little or no MMP-7 expression is detected in the normal kidney. However, its expression is markedly induced in human and animal models of kidney injury, which is primary controlled transcriptionally by β-catenin, the principal downstream mediator of canonical Wnt signaling [10]. We have previously shown that urinary matrix metalloproteinase-7 (uMMP-7) levels faithfully reflect renal Wnt/β-catenin activity, and the signal pathway is activated in AKI induced by ischemia-reperfusion injury or renal toxicity [10, 11].

Based on the above basic finding, we have recently validated that the postoperative uMMP-7 level predicts subsequent development of AKI and poor outcomes in patients who receive cardiac surgery [9]. Whether uMMP-7 can also serve as a biomarker forecasting the progression of AKI remains unknown. We therefore hypothesize that uMMP-7 might serve as a biomarker for predicting the progression of AKI which developed after cardiac surgery, and the combination of uMMP-7 and other reported biomarkers can further improve the prediction for AKI progression after cardiac surgery.

Here, we conducted a prospective, multicenter cohort study in 121 adult patients with stage 1 or 2 AKI after cardiac surgery to evaluate the utility of uMMP-7 and other reported biomarkers for predicting AKI progression during postoperative hospital stay.

2. Methods

2.1. Patients and Study Design. As previously described [9], we prospectively enrolled 398 adult patients who undergo elective cardiac surgery at six academic medical centers in China between September of 2013 and September of 2014. Of all 398 patients, only patients who developed initial stage 1 or stage 2 AKI (KDIGO criteria 2012 [12]) and with all tested biomarkers available at time of initial AKI diagnosis were included in this study. Patients were excluded if their initial AKI diagnosis was stage 3 since they would not progress further. The study was approved by the Institute Review Board of the National Clinical Research Center for Kidney Disease, and all participants provided written informed consent.

2.2. Procedure. We collected spot urine and blood samples before operation and at frequent intervals for 5 days after surgery. Urine sample collection and processing have been previously reported [9]. uMMP-7 and other reported biomarkers (urinary NGAL (uNGAL), urinary IL-18 (uIL-18)), as well as the urinary albumin to creatinine ratio (UACR), were measured at the day of initial AKI diagnosis. Serum creatinine was measured at least daily post operation and recorded for every patient throughout the hospital stay.

2.3. Biomarker Measurement. All of the biomarkers were measured in our central lab using a standard protocol to reduce the intra- or interassay variability. All the samples were labeled using study identification numbers without personal identifiers or clinical conditions. Urinary and MMP-7 levels were measured by an ELISA kit (DMP700; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Other reported biomarkers of renal injury, such as uNGAL, uIL-18, and UACR, were measured by available commercial ELISA kits according to the manufacturer's instruction.

2.4. Outcome Definitions. The primary outcome was the progression of AKI, defined as worsening of the KDIGO stage (from stage 1 to either stage 2 or stage 3 or from stage 2 to stage 3). Patients treated with acute dialysis at any point during hospitalization were defined as stage 3. Additional clinical outcomes were needed for acute dialysis, in-hospital mortality. Patients who died without progression were excluded from the primary analysis because death may have been a competing risk for progression for these patients.

2.5. Statistical Analyses. We performed the analyses with SPSS software (version 17.0). To compare continuous variables, we used a two-sample t-test or a Mann–Whitney U test. To compare categorical variables, we used the chi-squared or Fisher exact test. All tests were two-tailed, and $P < 0.05$ was considered significant.

As previously described [7], we categorized uMMP-7 tertile essentially as a continuous variable and then performed logistic regression on created variables. We determined the adjusted odds ratios (OR) of AKI progression with multiple logistic regression analysis. Due to the limited events, we adjusted for major risk factors for AKI progression after surgery, i.e., baseline estimated GFR, preoperative NYHA class, and cardiopulmonary bypass time, as well as percent change in serum creatinine from the baseline to the time of initial AKI diagnosis. uMMP-7 was also modeled as a continuous variable (log-transformed).

To compare the performance of uMMP-7 and other biomarkers, a conventional area under the receiver-operating characteristic (ROC) curve (AUC) was generated. To evaluate the utility of the biomarkers on risk classification, we determined the category-free net reclassification improvement (NRI) and the integrated discrimination improvement (IDI), as previously described [13, 14].

The performance of uMMP-7 for predicting AKI progression was internally validated by a bootstrap method with 1000 replications [15].

3. Results

3.1. Cohort Characteristics. A total of 121 patients who developed initial stage 1 or 2 AKI after cardiac surgery and with all tested biomarkers available at time of AKI were included in the final analysis.

Among 121 patients with AKI, 116 (95.8%) patients developed AKI within 72 hours post operation. A total of 28 patients (23.1%) progressed to a higher stage of AKI

Table 1: Preoperative characteristics of patients with and without AKI progression[a].

Variables	AKI progression		P
	Yes ($n = 28$)	No ($n = 93$)	
Age (y)	49.3 ± 10.9	51.2 ± 11.2	0.42
Male, n (%)	20 (71.4)	44 (47.3)	0.03
Diabetes, n (%)	2 (7.1)	7 (7.5)	0.99
Hypertension, n (%)	8 (28.6)	22 (23.7)	0.62
Congestive heart failure, n (%)	10 (35.7)	50 (53.8)	0.13
Preoperative NYHA class III or IV	5 (17.9)	21 (22.6)	0.78
Preoperative creatinine (μmol/L)	102.1 ± 40.3	80.3 ± 20.8	<0.001
Preoperative eGFR (mL/min/1.73 m^2)[b]	76.1 ± 25.3	86.7 ± 18.9	0.02
Preoperative medication, n (%)			
RAS inhibitors	12 (42.9)	23 (24.7)	0.09
Diuretics	25 (89.3)	89 (95.7)	0.35
Operative variables			
CABG alone, n (%)	1 (3.5)	2 (2.1)	0.55
Valve alone, n (%)	20 (71.4)	66 (70.9)	0.99
CABG and valve surgery, n (%)	2 (7.1)	5 (5.3)	0.66
CPB time (min)	163.6 ± 60.2	131.4 ± 51.8	0.006
Cross clamp time (min)	90.5 ± 40.6	86.3 ± 33.5	0.59

[a]AKI progression is defined as worsening of the AKI stage (stage 1 to either stage 2 or stage 3 or from stage 2 to stage 3). [b]Calculated by CKD-Epidemiology Collaboration equation 2009. Abbreviation: NYHA: New York Heart Association; eGFR: estimated glomerular filtration rate; RAS: renin-angiotensin system; CABG: coronary artery bypass grafting; CPB: cardiopulmonary bypass.

during their postoperative hospital stay (20 individuals progressed to stage 2 and 8 patients progressed to stage 3), 5 of 28 (17.9%) progressors received acute dialysis; 4 of 28 (14.3%) had AKI progression and subsequently died during their hospitalization, 93 patients (76.9%) persisted in stage 1 or 2 AKI, and none of them died or received acute dialysis during their postoperative hospital stay.

Table 1 shows the preoperative characteristics of 121 patients who developed or did not develop progressive AKI after cardiac surgery. There was no statistical difference in proportion of patients using RAS inhibitors or diuretics before surgery between those with or without AKI progression. Compared with those without AKI progression, patients with progressive AKI had lower preoperative eGFR and longer CPB time.

Table 2 compares postoperative characteristic and outcomes of patients with or without AKI progression. Patients with progressive AKI had a higher serum creatinine levels on the day of AKI diagnosis as compared to those without progressive AKI. Change of serum creatinine from the baseline (preoperative level) at the time of AKI diagnosis was also greater in patients with AKI progression. Levels of uMMP-7 and 3 previously reported urinary biomarkers (uIL-18, uNGAL, and UACR) were significantly higher in patients with progressive AKI as compared to those without. Patients with AKI progression had more adverse outcomes, such as receiving acute dialysis and in-hospital death, as compared with those without AKI progression (Table 2).

3.2. The Performance of uMMP-7 and Other Urinary Biomarkers for Predicting the Progression of AKI. The median of uMMP-7 and other biomarkers were significantly higher in patients with AKI progression compared to nonprogressors. There were graded responses across the tertiles of uMMP-7 level and the risk of AKI progression in the univariate model and remained statistically significant after adjusting for major clinical risk factors (Table 3). In the adjusted model, patients with the highest tertile of uMMP-7 (>7.8 μg/g Cr) had a 7.8-fold higher risk of AKI progression as compared with those with the lowest tertile of uMMP-7 (<2.7 μg/g Cr). When uMMP-7 was modeled as a continuous variable, higher levels of uMMP-7 were also associated with increased risk of progressive AKI in a multivariate model (OR per SD, 3.0; 95% CI, 1.4-6.2, $P = 0.002$).

uMMP-7 presented good performance for predicting progressive AKI after surgery, with an AUC of 0.80, greater than those of well-reported biomarkers (uIL-18, AUC 0.76; UACR, AUC 0.77; and uNGAL, AUC 0.65) (Figure 1(a)). The combination of uMMP-7 with uL-18 or UACR further improved the performance for predicting the progression of AKI, with AUCs of 0.84 (uMMP-7 and uIL-18) and 0.82 (uMMP-7 and UACR) (Figure 1(b)).

The performance of uMMP-7 was further confirmed by the bootstrap internal validation, in which the average AUC for predicting AKI progression (0.80; 95% CI 0.78-0.81) was comparable to that in the test cohort. Using the raw data without urinary creatinine correction, uMMP-7 also presented a comparable AUC (0.79, 95% CI 0.68-0.88) for predicting AKI progression.

3.3. The Improvement of the Risk Classification with the Injury Biomarkers to the Clinical Model. The addition of

Table 2: Postoperative characteristics and outcomes of patients with and without AKI progression[a].

	AKI progression		P
	Yes ($n = 28$)	No ($n = 93$)	
Time of AKI			
Within 3 days after surgery, n (%)	28 (100.0)	88 (94.6)	0.59
SCr on the day of AKI diagnosis (μmol/L)	184.2 ± 86.2	127.6 ± 35.9	<0.001
Change of SCr on the day of AKI (μmol/L)[b]	82.1 ± 79.1	46.8 ± 26.8	<0.001
Change of SCr on the day of AKI (%)[c]	90.7 ± 89.1	60.8 ± 35.4	0.01
Biomarkers on the day of AKI diagnosis			
uMMP-7 (μg/g Cr)	10.4 (5.6-25.7)	3.3 (1.3-7.3)	<0.001
uIL-18 (ng/g Cr)	305.5 (155.7-596.0)	110.9 (32.9-232.0)	<0.001
uNGAL (μg/g Cr)	146.6 (31.4-391.9)	50.8 (19.8-113.6)	0.01
UACR (mg/g Cr)	163.8 (84.1-312.9)	39.8 (16.2-100.7)	<0.001
Outcomes			
Acute dialysis, n (%)	5 (17.9)	0 (0.0)	<0.001
In-hospital death, n (%)	4 (14.3)	0 (0.0)	0.002

[a]AKI progression is defined as worsening of the AKI stage (stage 1 to either stage 2 or stage 3 or from stage 2 to stage 3). [b]Serum creatinine level on the day of AKI diagnosis minus baseline serum creatinine level. [c](SCr level on the day of AKI diagnosis-baseline SCr level)/baseline SCr level* 100%. Abbreviation: SCr: serum creatinine; uMMP-7: urinary matrix metalloproteinase-7; uIL-18: urinary interleukin-18; uNGAL: urinary neutrophil gelatinase-associated lipocalin; UACR: urinary albumin to creatinine ratio.

Table 3: Multivariate logistic regression analyses of uMMP-7 for predicting AKI progression[a].

uMMP-7	Cut points (μg/g Cr)	Progression (%)	Unadjusted OR (95% CI)	P	Adjusted OR[b] (95% CI)	P
Categorical						
Low (Tertile 1, $n = 40$)	<2.7	10.0	1.0 (referent)		1.0 (referent)	
Medium (Tertile 2, $n = 41$)	2.7-7.8	12.2	1.3 (0.3-5.0)	0.75	0.8 (0.2-4.2)	0.72
High (Tertile 3, $n = 40$)	>7.8	47.5	8.1 (2.4-27.2)	0.001	7.8 (1.9-36.0)	0.003
Continuous						
Per SD increase for lg transform	—	—	3.9 (1.8-8.2)	<0.001	3.0 (1.4-6.2)	0.002

[a]AKI progression is defined as worsening of the AKI stage (stage 1 to either stage 2 or stage 3 or from stage 2 to stage 3). [b]Adjusted for preoperative eGFR, preoperative NYHA class, CPB time, and change in postoperative serum creatinine from baseline at the time of AKI diagnosis.

uMMP-7 to the clinical risk factor model significantly improved risk classification for AKI progression, as evidenced by the net reclassification index (NRI) and the integrated discrimination improvement (IDI). Compared to reported biomarkers, uMMP-7 improved category-free NRI of 0.92, which was the greatest among those of all tested biomarkers (Table 4).

4. Discussion

In this prospective, multicenter study of adult patients who undergone cardiac surgery, we firstly showed that uMMP-7, measured at time of AKI diagnosis, is a novel biomarker for predicting the progression of AKI. A level of uMMP-7 > 7.8 μg/g Cr at time of AKI diagnosis denoted an 8-fold risk of AKI progression as compared to those with uMMP-7 < 2.7 μg/g after adjusting for major clinical risk factors. The performance of uMMP-7 for predicting progressive AKI post operation was good with an AUC of 0.80. The combination of uMMP-7 and IL-18 produced the greatest AUC for predicting progressive AKI. The addition of uMMP-7 to the clinical risk factor model significantly improved risk reclassification for AKI progression after cardiac surgery.

In recent years, there were studies demonstrated that renal injury biomarkers can detect acute kidney injury after cardiac surgery earlier than the increase of serum creatinine [8, 9]. Several biomarkers, such as IGF binding protein 7 and tissue inhibitors of metalloproteinase, have been approved by the US Food and Drug Administration as a first-of-a-kind test to help determine if surgical patients are at risk of developing AKI [16]. However, the identification of biomarkers that predict AKI progression in patients with established AKI has not been fully highlighted [8]. AKI progression after cardiac surgery is associated with increased risk of poor outcomes. In our cohort, patients who initially developed mild AKI and progressed to higher stages had mortality of 14% versus 0% in those who presented in original stages but not progressed, consistent with the previous report [5].

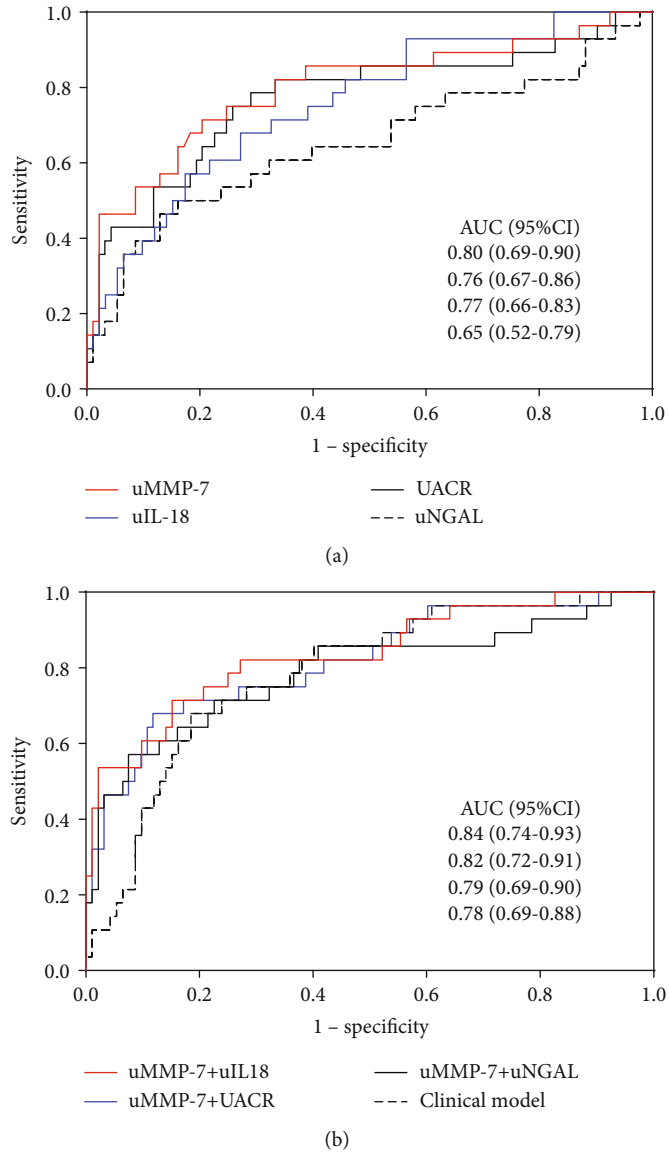

Figure 1: ROC analyses for predicting AKI progression. (a) The AUCs of urinary biomarkers (uMMP-7, uIL-18, UACR, and uNGAL), at the time of AKI diagnosis, for predicting AKI progression. (b) The performance of combination of urinary biomarkers, and clinical model alone, for predicting AKI progression.

Table 4: Risk reclassification of adding uMMP-7 and other biomarkers to the clinical model for predicting AKI progression[a].

Variables	Category-free NRI (95% CI)	*P*	Category-free NRI (95% CI) With events	*P*	Category-free NRI (95% CI) Without events	*P*	IDI (95% CI)	*P*
Clinical risk factors[b]	Referent		Referent		Referent		Referent	
Clinical risk factors+uMMP-7	0.92 (0.60-1.20)	<0.001	0.57 (0.25-0.90)	0.001	0.35 (0.15-0.54)	0.001	0.20 (0.12-0.28)	<0.001
Clinical risk factors+uIL-18	0.88 (0.50-1.16)	<0.001	0.50 (0.16-0.84)	0.006	0.38 (0.19-0.58)	<0.001	0.18 (0.09-0.27)	<0.001
Clinical risk factors+uNGAL	0.67 (0.47-0.87)	0.01	0.43 (0.07-0.78)	0.02	0.24 (0.04-0.44)	0.02	0.08 (0.02-0.14)	0.001
Clinical risk factors+UACR	0.88 (0.50-1.16)	<0.001	0.48 (0.25-0.80)	0.007	0.40 (0.21-0.62)	<0.001	0.18 (0.09-0.27)	<0.001

[a]AKI progression is defined as worsening of the AKI stage (stage 1 to either stage 2 or stage 3 or from stage 2 to stage 3). [b]Comprised of preoperative eGFR, CPB time, and change in postoperative serum creatinine from baseline at the time of AKI diagnosis. Abbreviation: NRI: net reclassification improvement; IDI: integrated discrimination improvement; CI: confidence interval.

It is therefore critical to identify patients at highest risk of AKI progression so as to guide prognosis and management decisions. There are several studies that reported that biomarkers, measured at time of AKI clinical diagnosis, predicted AKI development after cardiac surgery [4, 5]. A recent study tested the ability of 32 biomarkers to predict worsening of renal function in patients with AKIN stage 1 AKI after cardiac surgery [4]. They found that uIL-18 was the best predictor of worsening AKI. In a larger study from the Translational Research Investigating Biomarker Endpoints-AKI consortium, uIL-18, UACR, and uNGAL measurement at the time of AKI diagnosis predicted the progression of AKI in adults after cardiac surgery [5]. In our study, elevation of uMMP-7 is an independent predictor of progressive AKI after cardiac surgery after adjusting for major preoperative and intraoperative risk factors and provides good performance for predicting AKI progression. Furthermore, adding uMMP-7 to the clinical risk model significantly improves risk reclassification for AKI progression, suggesting that early measurement of uMMP-7 at time of AKI might be helpful to accurately identify patients at increased risk for AKI progression, and may offer clinicians an earlier time window to halt or reverse ongoing kidney injury.

The potential role of elevated renal MMP-7 in human AKI progression is waiting for exploration. uMMP-7 is a marker faithfully reflecting intrarenal Wnt/beta-catenin activity and dependably mirrors its expression in renal parenchyma, particularly in the tubular epithelium [10]. Tubular MMP-7 expression is significantly induced after renal ischemia-reperfusion injury [17]. Recent experimental data found that sustained Wnt/beta-catenin activating after ischemia-reperfusion injury might drive kidney injury progression [11], suggesting that uMMP-7 could be selected as a marker of AKI progression.

To further enhance the ability of biomarkers for predicting AKI progression after cardiac surgery, carefully selecting and combining biomarkers might be a better approach for greater use. Urinary IL-18, an inflammation marker of injury, has been consistently reported as a predictive biomarker for progressive AKI after cardiac surgery [4, 5]. In our study, combining uIL-18 and uMMP-7 produced the greatest AUC (0.84) compared with combining uNGAL or UACR, supporting a multibiomarker approach which might further improve the predictive ability of biomarker for AKI progression after cardiac surgery [8].

Our study has the following strengths. First, it is a multicenter, prospective cohort study and relied on standardized AKI staging criteria (KDIGO) that are currently used in the international renal community. Second, serum creatinine was measured everyday post cardiac surgery, which allowed us to precisely define AKI and determine AKI progression. Third, we simultaneously measured previously reported biomarkers and assessed the predictive performance of uMMP-7 with other established biomarker for predicting AKI progression in the setting of cardiac surgery, which directly compares the predictive ability of biomarkers alone or in combination. This study also had limitations. Urinary creatinine excretion is not at a steady state during AKI; 24 h urinary excretion of MMP7 would be more meaningful. The number of primary outcome was relatively small, and all patients were Chinese adults; validation studies from other ethnic populations are warranted.

In conclusion, uMMP-7 measured at time of AKI clinical diagnosis predicts AKI progression. Adding uMMP-7 to the clinical risk factor model may be used as a noninvasive approach to identify patients that are at high risk for progressive AKI after cardiac surgery, which may facilitate patient counseling and optimize management in the setting of cardiac surgery.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' Contributions

Fan Fang and Weihong Luo contributed equally to this study.

Acknowledgments

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References

[1] M. H. Rosner and M. D. Okusa, "Acute kidney injury associated with cardiac surgery," *Clinical Journal of the American Society of Nephrology*, vol. 1, no. 1, pp. 19–32, 2006.

[2] G. Zanardo, P. Michielon, A. Paccagnella et al., "Acute renal failure in the patient undergoing cardiac operation. Prevalence, mortality rate, and main risk factors," *The Journal of Thoracic and Cardiovascular Surgery*, vol. 107, no. 6, pp. 1489–1495, 1994.

[3] K. C. Leung, M. Tonelli, and M. T. James, "Chronic kidney disease following acute kidney injury–risk and outcomes," *Nature Reviews. Nephrology*, vol. 9, no. 2, pp. 77–85, 2013.

[4] J. M. Arthur, E. G. Hill, J. L. Alge et al., "Evaluation of 32 urine biomarkers to predict the progression of acute kidney injury after cardiac surgery," *Kidney International*, vol. 85, no. 2, pp. 431–438, 2014.

[5] J. L. Koyner, A. X. Garg, S. G. Coca et al., "Biomarkers predict progression of acute kidney injury after cardiac surgery," *Journal of the American Society of Nephrology*, vol. 23, no. 5, pp. 905–914, 2012.

[6] P. Susantitaphong, D. N. Cruz, J. Cerda et al., "World incidence of AKI: a meta-analysis," *Clinical Journal of the American Society of Nephrology*, vol. 8, no. 9, pp. 1482–1493, 2013.

[7] C. Chen, X. Yang, Y. Lei et al., "Urinary biomarkers at the time of AKI diagnosis as predictors of progression of AKI among patients with acute cardiorenal syndrome," *Clinical Journal*

of the American Society of Nephrology, vol. 11, no. 9, pp. 1536–1544, 2016.

[8] C. R. Parikh, S. G. Coca, H. Thiessen-Philbrook et al., "Postoperative biomarkers predict acute kidney injury and poor outcomes after adult cardiac surgery," *Journal of the American Society of Nephrology*, vol. 22, no. 9, pp. 1748–1757, 2011.

[9] X. Yang, C. Chen, S. Teng et al., "Urinary matrix metalloproteinase-7 predicts severe AKI and poor outcomes after cardiac surgery," *Journal of the American Society of Nephrology*, vol. 28, no. 11, pp. 3373–3382, 2017.

[10] W. He, R. J. Tan, Y. Li et al., "Matrix metalloproteinase-7 as a surrogate marker predicts renal Wnt/β-catenin activity in CKD," *Journal of the American Society of Nephrology*, vol. 23, no. 2, pp. 294–304, 2012.

[11] L. Xiao, D. Zhou, R. J. Tan et al., "Sustained activation of Wnt/β-Catenin signaling drives AKI to CKD progression," *Journal of the American Society of Nephrology*, vol. 27, no. 6, pp. 1727–1740, 2016.

[12] J. A. Kellum, N. Lameire, P. Aspelin et al., "Kidney disease: improving global outcomes (KDIGO) acute kidney injury work group. KDIGO clinical practice guideline for acute kidney injury," *Kidney International Supplements*, vol. 2, no. 1, pp. 1–138, 2012.

[13] N. R. Cook, "Statistical evaluation of prognostic versus diagnostic models: beyond the ROC curve," *Clinical Chemistry*, vol. 54, no. 1, pp. 17–23, 2008.

[14] M. J. Pencina, R. B. D'Agostino Sr., R. B. D'Agostino Jr., and R. S. Vasan, "Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond," *Statistics in Medicine*, vol. 27, pp. 157–172, 2008.

[15] B. Efron and R. J. Tibshirani, *An Introduction to the Bootstrap*, CRC Press, Boca Raton, Florida, 1994.

[16] K. J. Gunnerson, A. D. Shaw, L. S. Chawla et al., "TIMP2* IGFBP7 biomarker panel accurately predicts acute kidney injury in high-risk surgical patients," *Journal of Trauma and Acute Care Surgery*, vol. 80, no. 2, pp. 243–249, 2016.

[17] D. Zhou, Y. Li, L. Lin, L. Zhou, P. Igarashi, and Y. Liu, "Tubule-specific ablation of endogenous β-catenin aggravates acute kidney injury in mice," *Kidney International*, vol. 82, no. 5, pp. 537–547, 2012.

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Research Article

Inflammation-Related Patterns in the Clinical Staging and Severity Assessment of Chronic Kidney Disease

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Chronic kidney disease (CKD) is an irreversible loss of kidney function, and it represents a major global public health burden due to both its prevalence and its continuously increasing incidence. Mineral bone disorders (MBDs) constitute a hallmark of CKD, and alongside cardiovascular complications, they underlie a poor prognosis for these patients. Thus, our study focused on novel CKD biomarker patterns and their impact on the clinical staging of the disease. As a first testing approach, the relative expression levels of 105 proteins were assessed by the Proteome Profiler Cytokine Array Kit for pooled CKD stage 2–4 serum samples to establish an overall view regarding the proteins involved in CKD pathogenesis. Among the molecules that displayed significant dysregulation in the CKD stages, we further explored the involvement of Dickkopf-related protein 1 (Dkk-1), a recognised inhibitor of the Wnt signalling pathway, and its crosstalk with $1,25OH_2D_3$ (calcitriol) as new players in renal bone and vascular disease. The serum levels of these two molecules were quantified by an ELISA (76 samples), and the results reveal decreasing circulating levels of Dkk-1 and calcitriol in advanced CKD stages, with their circulating expression showing a downward trend as the CKD develops. In the next step, we analysed the inflammation and MBD biomarkers' expression in CKD (by xMAP array). Our results show that the molecules involved in orchestrating the inflammatory response, interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα), as well as the mineral biomarkers osteoprotegerin (OPG), osteocalcin (OC), osteopontin (OPN), and fibroblast growth factor 23 (FGF-23), correlate with Dkk-1 and calcitriol, raising the possibility of them being potential useful CKD biomarkers. These results reveal the impact of different biomarker patterns in CKD staging and severity, thus opening up novel approaches to be explored in CKD clinical management.

1. Introduction

Chronic kidney disease (CKD) represents a major global disease that covers all degrees of injured renal function, with a rising incidence and prevalence of kidney failure resulting in poor outcomes and high economic costs. According to the Kidney Disease Improving Global Outcomes (KDIGO) 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease: Mineral and Bone Disorder (CKD-MBD), the disease is defined as "abnormalities of the kidney structure or function, present for more than 3 months, with

implications for health" [1]. The characteristic features of CKD are the progressive and irreversible loss of renal function, which results in extensive kidney damage, leading unconditionally to end-stage renal disease (ESRD). Over the last 10 years, CKD has reached epidemic proportions, with a constant increase in terms of both prevalence and incidence, and it has been classified by the Global Burden of Disease Study as "the 12th most common cause of death, accounting for 1.1 million deaths worldwide." Overall, its poor prognoses ranked CKD as "the 17th leading cause of global year loss of life and the 3rd largest increase of any major cause of death" [2, 3].

Cardiovascular disease (CVD) is noted as the main cause of morbidity and mortality in these patients, while CKD is considered an accelerator of cardiovascular events and an independent risk factor for CVD. It was also shown that all CKD stages are accompanied by an elevated risk of cardiovascular complications and a decreased quality of life [4].

The causes of high cardiovascular mortality related to CKD have been attributed in part to CKD-MBD syndrome, which generates a unique environment that accelerates vascular calcification (VC)—the pathological deposition of calcium phosphate in the vasculature's medial layer. Even in the early CKD stages, the systemic mineral metabolism and bone composition begin to alter; thus, the dysregulation of mineral metabolism is considered a key player in CKD pathophysiology.

An imbalance in the kidney-vascular-bone axis, a multifaceted active process, is induced by mineral metabolism disorders and also by local inflammation; nevertheless, the most extensive mineral disorders are experienced by patients suffering from CKD [5].

The discovery of Wnt inhibitors, among them Dickkopf-related protein 1 (Dkk-1), released during renal repair as crucial components of mineral bone disorder (MBD) pathogenesis, suggests that additional pathogenic factors need to be explored [6, 7].

Elucidating the signalling pathways involved in vascular smooth muscle cell calcification holds the promise of being able to unravel novel therapeutic approaches counteracting the progression of MBDs in CKD.

Various factors mediate the VC mechanisms including disturbances in the serum calcium/phosphate balance, systemic and local inflammation, the receptor activator of nuclear factor kappa B (RANK)/RANK ligand (RANK-L)/osteoprotegerin (OPG) triad, aldosterone, microRNAs, osteogenic transdifferentiation, and the effects of vitamins [8]. The emerging role of 1,25-dihydroxyvitamin D_3 (calcitriol, $1,25OH_2D_3$) in CKD has been extensively explored, since vitamin D deficiency/insufficiency is known to be common among patients with CKD or in those undergoing dialysis. Vitamin D has pleiotropic effects on the immune, cardiovascular, and neurological systems, and many extrarenal organs have the enzymatic capability to convert $25OHD_3$ to $1,25OH_2D_3$. It was also hypothesised that serum $1,25OH_2D_3$ and 25OHD expressions tend to positively correlate, together with the renal function, as well [9].

Persistent low-grade inflammation is currently considered an essential part of CKD and as a traditional risk factor for renal pathology, hugely contributing to the development of all-cause mortality in these patients [10]. The role of proinflammatory cytokine overexpression inside the renal patient's landscape has drawn considerable attention, and various studies have explored the potential link between inflammatory status and renal function decline [11, 12]. A challenging theory regarding the direct consequence of inflammation on the progression of both CKD and CVD was developed based on the supposition of this association between markers of inflammation and an estimated glomerular filtration rate (eGFR) imbalance [13].

Despite the accessibility to the studies published in the past few years, the KDIGO Guideline Committee underlines the lack of strong clinical proof, emphasizing the critical role of understanding the mechanisms underlying the disease's development, yet stressing the need for comprehensive, accurate clinical trials in this direction [14].

Considering the aforementioned aspects, in this study, the correlation between the severity of CKD and inflammatory factors, MBD biomarkers, and other novel biomarkers with an impact on CKD's pathophysiology was investigated to reveal potential proteome patterns that better characterise the condition characteristic of each stage of CKD.

2. Materials and Methods

2.1. Patients and Samples

2.1.1. Study Population. We included 56 patients in our cross-sectional study who were diagnosed with CKD according to the KDIGO Guidelines alongside 20 normal controls. The CKD patients were divided into three groups based on the CKD staging criteria as follows: 16 patients with CKD stage 4 (25% female and 75% male; mean age 63 ± 14.8), 26 with CKD stage 3 (31% female and 69% male; mean age 68 ± 8.5), and 14 with CKD stage 2 (29% female and 71% male; mean age 65 ± 10.3). Written informed consent was obtained from all subjects prior to their inclusion in the study according to the Helsinki Declaration and Ethics Committee that approved this study.

Patients with acute infections, acute heart failure and significant heart valvular disease, chronic use of glucocorticoids and immunosuppressive agents, and known malignancy were excluded from our study. In addition, in order to avoid the potential bias, patients undergoing vitamin D synthetic analogue treatment were also excluded.

2.1.2. Clinical and Biochemical Assessment. On the day the blood samples were collected, clinical and anthropometric data were gathered: age, sex, weight, height, medical history, and concomitant treatment. Laboratory tests were performed on admission, namely, haemoglobin, haematocrit, serum creatinine, urea, uric acid, glucose, total cholesterol, triglycerides, alkaline phosphatase, phosphate, calcium, albumin, and fibrinogen. The eGFR was calculated based on the CKD-epidemiology collaboration (EPI) equation. Urinary protein excretion was determined from a 24 h urine sample.

The blood samples were harvested the morning after a 12 h fast. After a standard centrifugation, the serum was aliquoted and stored at $-80^{\circ}C$ pending further analysis.

2.2. Human Dot-Blot Proteome Profiler. Semiquantitative immunodetection of serum cytokines, chemokines, growth factors, angiogenesis markers, and other soluble proteins was performed using the immuno-dot-blot method in the Proteome Profiler Human XL Cytokine Array Kit (ARY022B, R&D Systems, Inc., Abingdon, UK). A number of 105 captured antibodies, along with reference controls, were spotted in duplicate on nitrocellulose membranes and incubated overnight with 100 mL of pooled serum samples. Each of the four pools was obtained by mixing the serum samples from CKD patients in stages 4, 3, and 2, respectively; the 4th pool was assigned to control sera. The protocol recommended by the manufacturer was followed accordingly. The membranes were incubated with biotinylated detection antibodies, streptavidin-horseradish peroxidase (HRP), and chemoluminescent detection reagents. Chemiluminescence signals, corresponding to the amount of protein bound, were detected using the MicroChemi 4.2 System (DNR Bio-Imaging Systems, Israel), and the intensity of the chemiluminescence signals (pixel densities) was measured using ImageJ 1.42 software (National Institute of Health, Bethesda, MD, USA). For each measured analyte, the average signal of the duplicate spots was determined and normalised to the average signal of the reference spots after being corrected with the background signal.

2.3. ELISA Immunoassay. Dkk-1 serum levels were assessed using the Quantikine ELISA Human Dkk-1 Immunoassay Kit (R&D Systems, Inc., USA) according to the manufacturer's protocol. The quantitative determination of the calcitriol ($1,25OH_2D_3$ (1,25-dihydroxyvitamin D_3)) serum levels was made using the EIAab ELISA General Calcitriol Kit (Wuhan EIAab Science Co., Ltd., China), and the manufacturer's instructions were followed accordingly. The optical densities were measured using an Anthos Zenyth 3100 Microplate Multimode Detector.

2.4. Luminex xMAP Array Analysis. The Luminex xMAP array procedure was performed according to the manufacturer's instructions. The serum levels of the 6-plex analytes were simultaneously quantified on the Luminex 200 multiplexing platform. The Luminex xMAP array technique is based on proprietary colour-coded microspheres coated with specific capture antibodies. After the analytes from the serum samples were captured by the bead cocktail, a biotinylated detection antibody was added. The reaction mixture was then incubated with the reporter molecule conjugate (streptavidin-phycoerythrin (SA-PE)) to complete the reaction on the surface of the microspheres. After the reaction steps had been completed, the microspheres were passed rapidly through a red laser which excited the internal dyes, thus identifying each unique microsphere set. The green laser excited PE, the fluorescent dye on the reporter molecule, which was directly correlated with the amount of analyte found in the sample. All the acquired data was processed by high-speed digital-signal processors and by xPONENT 3.1 software, generating results expressed in pg/mL.

Cytokine levels and BMD biomarkers were assayed using the MILLIPLEX MAP Human Bone Magnetic Bead Panel Kit (Merck-Millipore, Billerica, MA, USA), which comprises a cocktail of six analytes: proinflammatory cytokines IL-6 and TNF-α and the MBD biomarkers OPG, osteocalcin (OCN), osteopontin (OPN), and fibroblast growth factor 23 (FGF-23).

For all the biological specimens, duplicate samples were used and their average concentrations were taken into consideration for further statistical analysis.

2.5. Statistical Analysis. As a first statistical approach, we applied the Kolmogorov-Smirnov and D'Agostino and Pearson normality tests to all the CKD and control samples under analysis. The Kolmogorov-Smirnov test was used to evaluate the normality of the data distribution. The groups presented with a nonnormal distribution ($p < 0.0001$); therefore, nonparametric statistical tests were used for further analysis. The groups were not homogeneous in terms of age and gender, but according to the results obtained after applying the Chi-square test, they did not influence the level of the analysed molecules; age was expressed as the mean ± SD. The differences between the variables were analysed using the Kruskal-Wallis test (a one-way analysis of variance) followed by a Bonferroni post hoc test to compare the results inside the different CKD stage groups. The Chi-square test for trends was applied to reveal the differences in molecule expression between the various CKD stages. The differences between the nominal variables were analysed using Chi-square tests (r, p). A value of $p < 0.05$ was considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). Spearman's correlation analysis was used to evaluate the correlations between the analysed markers (r, p). GraphPad Prism version 5 software for Windows was used for the statistical analysis.

3. Results and Discussion

3.1. Proteome Profiler for CKD Clinical Staging by Dot-Blot Array Assessment. An overall perspective on the multiple proteins that are differentially expressed in the CKD stages and thus potentially influence CKD's pathophysiology was gained by performing semiquantitative dot-blot immunodetection [15, 16]. Out of 105 molecules included in the Proteome Profiler Human XL Cytokine Array Kit, 24 relevant molecules were identified as expressing significant levels in CKD patients versus the control group. At first glance, the dot-blot analysis revealed that molecules orchestrating the inflammatory response were significantly overexpressed in CKD; moreover, the multianalyte screening showed different patterns of expression depending on the CKD stage (as illustrated in Figure 1). The integrated relative pixel density of these molecules trended towards a progressive pattern of expression, exhibiting gradual amounts depending on the stage of renal disease. The most significant expression level for proteins was identified in CKD stage 4. Among the proteins that displayed a significant fold change versus the control (about a 1.5-fold change), markers for inflammatory response were identified, reflecting the high significance of the inflammatory component in CKD. Among them, IL-6, IL-8, IL-12, IL-18, interferon gamma (IFN-γ), the regulated upon activation normal T-cell expressed and secreted

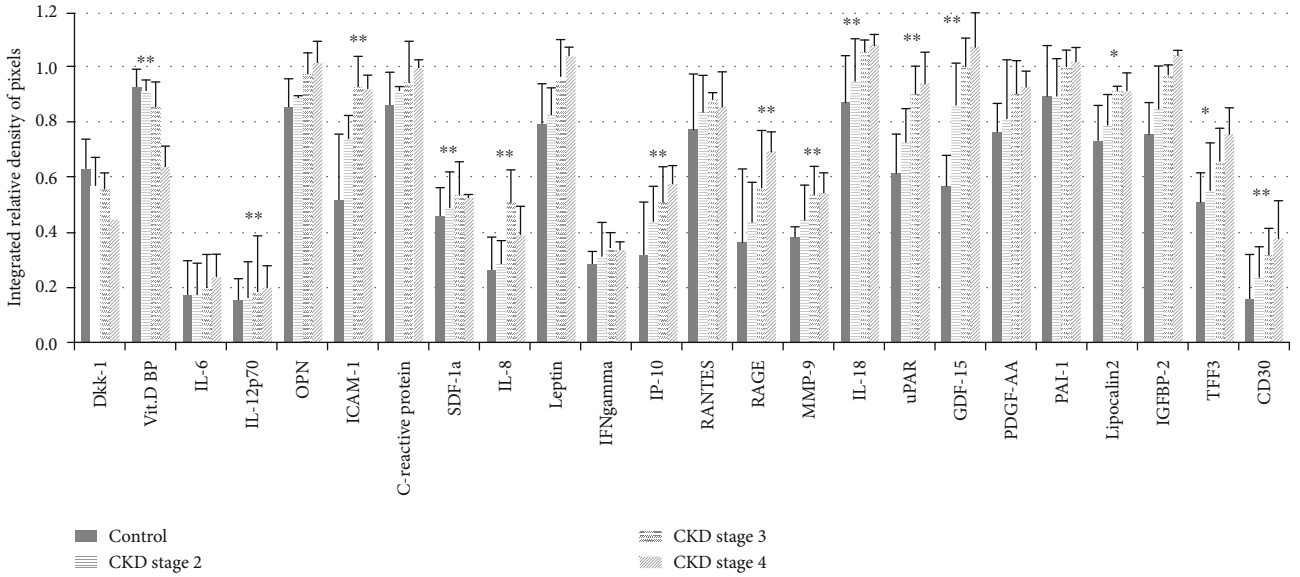

Figure 1: Original dot-blot membranes of the Proteome Profiler corresponding to different CKD stages and the control. The representative molecules that exhibited significant fold changes versus the control and were the subject of further analysis have been marked accordingly.

(RANTES), the receptor for advanced glycation end products (RAGE), intercellular adhesion molecule 1 (ICAM-1), inducible protein 10 (IP-10), plasminogen activator inhibitor 1 (PAI-1), platelet-derived growth factor (PDGF), and others were identified as having a place in the CKD proteome pattern, as shown in Figure 1.

Persistent, low-grade inflammation constitutes a common feature of the disease, which accompanies CKD from its onset [17]. Inflammatory biomarkers such as C-reactive protein and IL-6 are known to independently predict mortality in these patients. The origins of inflammation in kidney disease are multifactorial, including the imbalance between proinflammatory increased production, induced on the one hand by various sources of inflammatory stimuli (oxidative stress, acidosis, comorbidities, genetic and epigenetic influences, etc.) and on the other hand by their insufficient elimination due to impaired glomerular filtration [18]. IL-6 hastens the development of CKD not only by aggravating kidney injury but also by initiating its complications, especially the cardiovascular ones. It is well established that IL-6 initiates the endothelial injury mostly by reducing endothelial nitric oxide synthase and adiponectin (an antiatherogenic adipokine) expression, thus contributing to the increased incidence of cardiovascular events in CKD patients. Taken together, an increased IL-6 level not only is a consequence of CKD but also acts as a trigger for CKD-related complications [19].

Mediators of inflammation have been shown to be at high levels in CKD patients. IL-12 and IL-18 are elevated during the earlier stages of CKD, and the association with eGFR suggests that IL-18 is mainly dependent upon renal clearance, as suggested by Yong et al. [20].

The urokinase receptor system, a key regulator at the intersection between inflammation, immunity, and coagulation [21], has also been shown to significantly increase in CKD patients. Nuclear factor kappa B (NF-κB), a pivotal mediator of inflammatory responses through triggering the prototypical proinflammatory signalling pathway, appears to mediate renal inflammation in different cell types including renal cells, innate immune cells, and lymphocytes [22, 23]. It was shown that NF-κB also controls several genes involved in inflammation, and RAGE (an advanced glycation end-product-specific receptor) itself seems to be upregulated by NF-κB [24].

The pleiotropic cytokine OPN is increased in early CKD stages, and its circulatory level increases with the severity of the disease stage. OPN is an important factor in bone remodelling, as it is involved in the pathogenesis of both kidney and cardiovascular diseases. Barreto et al. reported a positive correlation between OPN levels and the clinical outcomes of CKD patients depending on their inflammatory status [25].

The interplay between different proteins involved in inflammation and the MBD profile is depicted in Figures 1 and 2. Among the molecules that exhibited significant downregulation in CKD stage 4 versus the control (with about a 1.5-fold change, $p < 0.05$—illustrated in Figure 3), Dkk-1 and vitamin D binding protein (vit D BP) showed the highest potential and were chosen for further analyses. The proinflammatory cytokine IL-6 and the MBD biomarker OPN, with significant increases in CKD, were also subject to further analyses.

3.2. Dkk-1 Was Negatively Correlated with CKD Clinical Staging. Recent studies emphasize the close connection between CKD and cardiovascular complications, as well as the presence of a dysregulated Wnt signalling pathway in CVD.

[26, 27]. Based on these facts, we explored the circulating expression of Dkk-1, a recognised inhibitor of the Wnt–β-catenin signalling pathway, in modulating the renal disease course. Targeting the Wnt signalling cascade aligns with innovative therapeutic CVD strategies [28, 29].

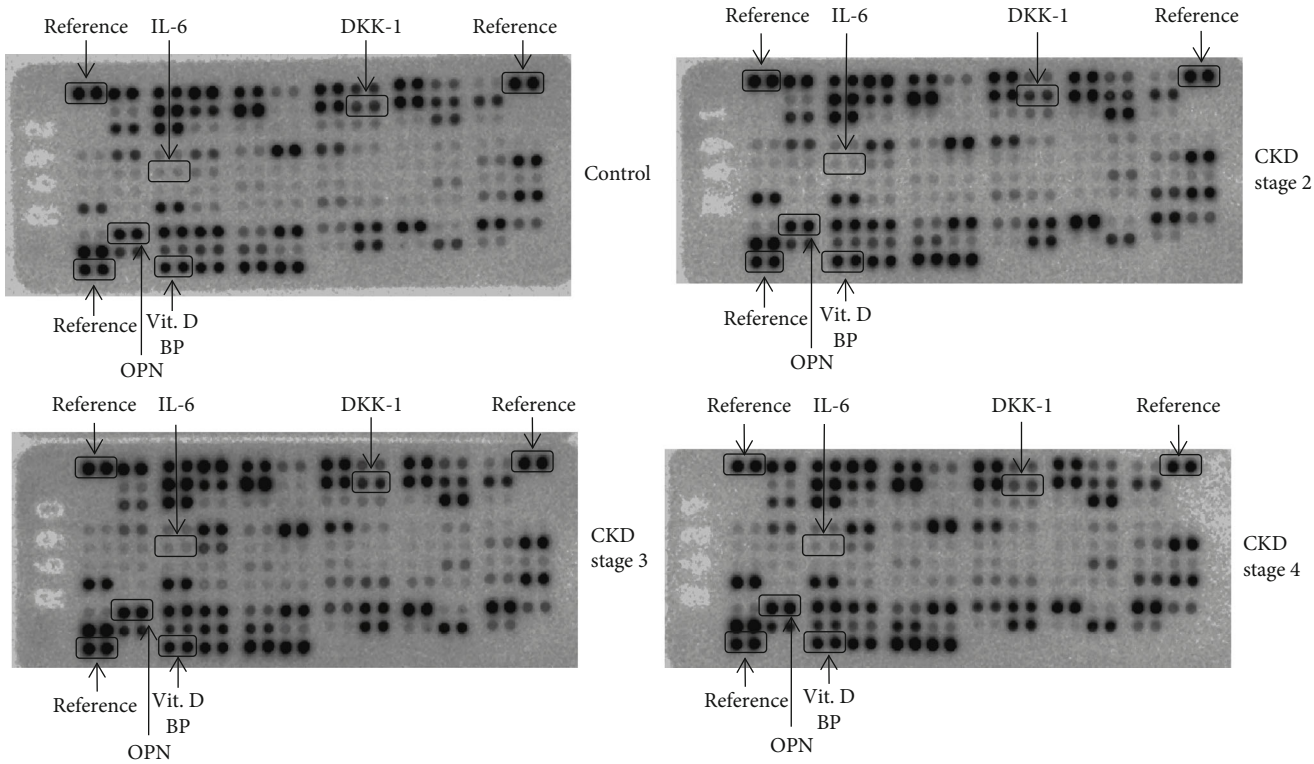

Figure 2: Serum protein profiling in CKD stages 2–4 versus the control. The integrated relative density of the pixels was calculated for each molecule after normalisation to the average signal of the reference spots. The molecules showed an ascending trend of expression according to the severity of the disease; Dkk-1 and vit D BP showed a descending trend.

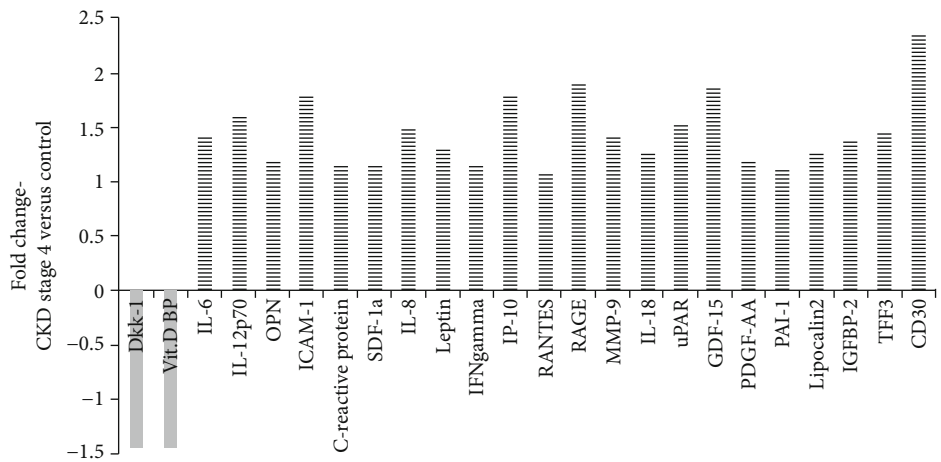

Figure 3: The fold change in protein expression in CKD stage 4 versus the control. The average for the control group was established at 1.0, and for each analysed molecule, the fold change was expressed as the CKD stage 4/control ratio.

The significant downregulation of Dkk-1 in CKD stage 4, determined via a dot-blot analysis, was further confirmed by running a quantitative ELISA. Our results showed a statistically significant decreased expression of Dkk-1 in CKD patients compared to the control group ($p < 0.05$, Figure 4).

Relative serum Dkk-1 levels decreased even in the early stages of CKD, with a 1.05-fold decrease in stage 2 versus the control and a 1.3-fold decrease in stage 3. Dkk-1 circulating levels showed a downward trend, culminating in stage 4, where a significant 2.36-fold decrease was recorded versus the control. Recent studies have also reported that serum Dkk-1 levels were lower in CKD patients as compared with controls and that Dkk-1 levels had a tendency to decrease with the progressive development of CKD [30]. Interestingly, Behets et al. reported lower levels in CKD patients than in the controls, but Dkk-1 levels were not associated with the laboratory parameters of mineral metabolism. Since these

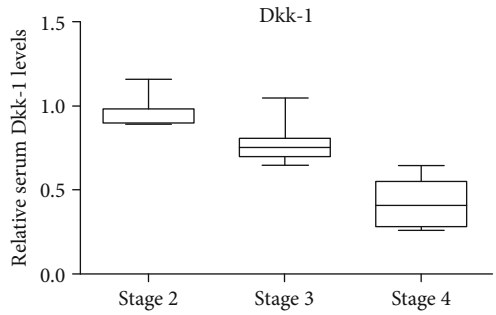

Figure 4: Dkk-1 fold change expression in CKD stages 4, 3, and 2 versus the control, assessed by ELISA.

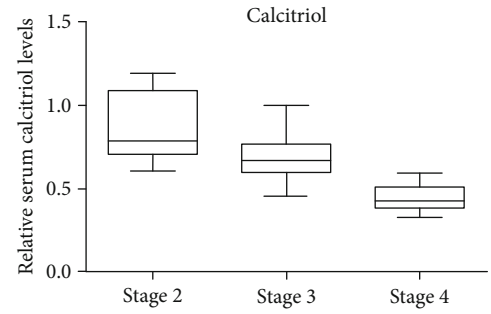

Figure 5: Calcitriol fold change expression in CKD stages 4, 3, and 2 versus the control, assessed by ELISA.

correlations were applied only to haemodialysis patients, it was hypothesised that Dkk-1 targeted different regulatory mechanisms inside the Wnt–β-catenin signalling pathway [31]. Thus, Dkk-1 seems to have distinct effects depending on the cell type, which is in line with the different effects of Wnt–β-catenin signalling. Increasing evidence indicates that the Wnt–β-catenin signalling pathway has important roles in skeletal development and bone mass equilibrium. It was found that Wnt activation increases bone formation and reduces bone desorption; therefore, a disturbed Wnt–β-catenin signalling pathway may be involved in CKD-MBD pathophysiology [32, 33]. Since VC is a hallmark feature of chronic inflammatory disorders, it has been shown that CKD aggravates vascular inflammation [34]. In a study conducted by Jang et al., the role of Dkk-1 in mediating the inflammatory response was investigated, thus exploring the implications of the Wnt signalling pathway in promoting immune responses or inflammation by triggering NF-κB activity. In this study, lipopolysaccharide- (LPS-) induced inflammatory responses were found to be prevented by Dkk-1 in a dose-dependent manner in human bronchial epithelial cells and human umbilical vein endothelial cells (HUVEC). Therefore, LPS-induced expression of the proinflammatory cytokines IL-6 and IL-8 was inhibited by Dkk-1. Other proinflammatory genes such as TNF-α and IL-1β were also downregulated by Dkk-1, a secreted Wnt antagonist [35].

Exploring the potential of the Wnt–β-catenin signalling pathway inhibitor Dkk-1 in predicting the severity of CKD and elucidating its role in CKD-MBD pathophysiology is thus a promising strategy for further studies.

3.3. Calcitriol Levels Decrease with Increasing CKD Stage. A vitamin D deficiency is a common condition associated with kidney disease. Many clinical studies have highlighted how a vitamin D deficiency is an important risk factor for CKD patients [36, 37].

Since dot-blot screening revealed significantly decreased levels of vitamin D BP, we thus measured the most active vitamin D metabolite in the kidneys: calcitriol (1,25-dihydroxyvitamin D_3 ($1,25OH_2D_3$)).

Experimental studies have established that calcitriol and vitamin D receptors are decisive regulators of the heart in terms of structure and function. In addition, clinical studies have correlated vitamin D deficiency with CVD. Emerging evidence has highlighted that calcitriol is significantly involved in CVD-related signalling pathways, particularly in the Wnt signalling pathway [38].

Our results revealed that relative serum calcitriol levels started to decrease even in the early CKD stages, showing a 1.15-fold decrease in stage 2 compared to the control condition, a 1.5-fold decrease in stage 3, and a 2.24-fold decrease in stage 4, respectively (as depicted in Figure 5), thus gradually decreasing as the disease develops. In the early CKD stages, the physiologic FGF-23 secretion from the osteocytes causes inhibition of 1-α-hydroxylase and stimulation of 24-hydroxylase in proximal renal tubules, thereby decreasing calcitriol production. As CKD evolves, the decrease in the functioning nephron mass combined with hyperphosphatemia and high FGF-23 levels also results in calcitriol deficiency [6, 39]. Since inflammation has emerged to be at the core of CKD pathophysiology, it was also hypothesised that vitamin D has a potential role in modulating inflammatory cytokines and oxidative stress, but the molecular mechanisms still remain unclear [40]. Recent studies have shown that vitamin D supplementation among CKD patients undergoing dialysis had beneficial effects on several genes related to inflammation and oxidative stress. The downward trend in calcitriol concentrations in the CKD groups could be related to various inflammatory and MBD factors, thus providing the basis for future clinical assessments.

3.4. Multiplexing Showed Inflammatory and Mineral Bone Disorder Biomarker Levels to Be Positively Correlated with Disease Severity. Among the many contributors to CKD's poor prognosis, systemic low-grade inflammation is one of the major players with an impact on the uremic phenotype in CKD. This chronic condition is fuelled by several independent mechanisms, among which the mediators of inflammation, IL-6 and TNFα, play important roles. We have simultaneously quantified the serum levels of IL-6 and TNFα using the Luminex multiplexing xMAP array platform, assaying via a preconfigured cytokine kit. Our results revealed that relative serum IL-6 levels started to increase in CKD early stage 2, showing a 1.9-fold change compared to the control, with an ascending trend, presenting with a 6.3-fold increase in stage 3 and an 11-fold increase in stage 4 (Figure 6(a)). Analysing the circulating

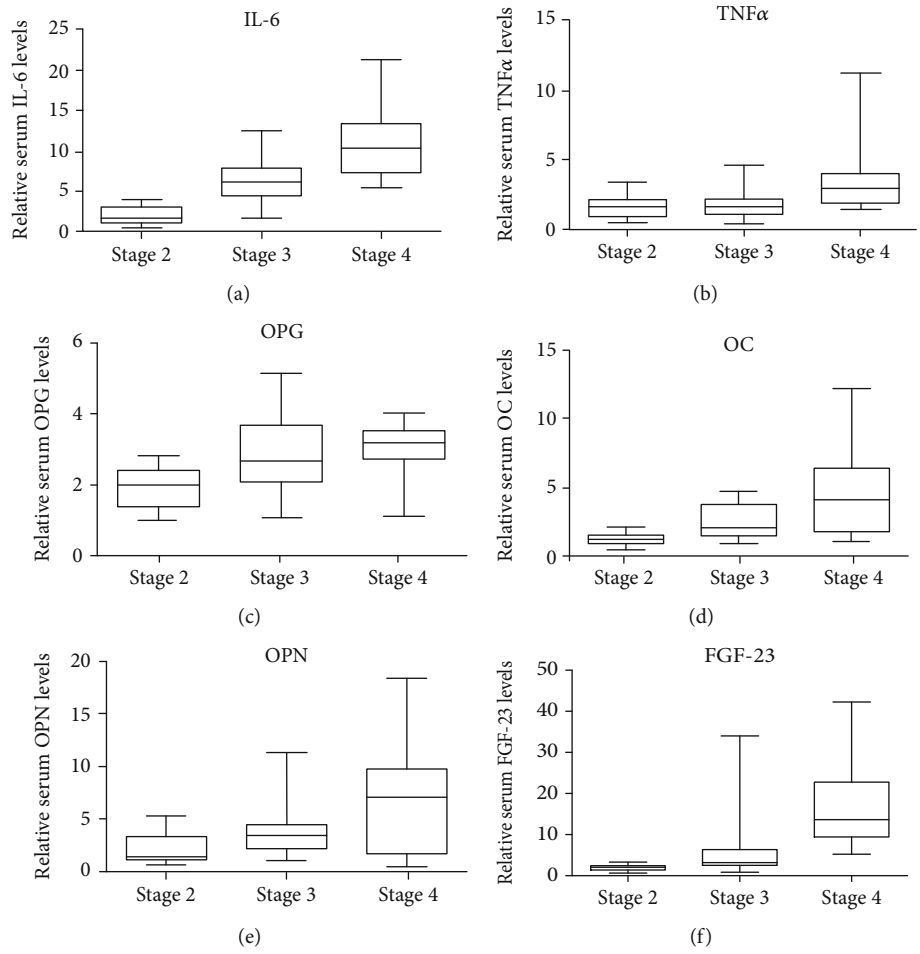

Figure 6: Fold change in serum IL-6 (a), TNFα (b), OPG (c), OC (d), OPN (e), and FGF-23 (f) expressions in CKD stages 4, 3, and 2 versus the control, assessed by xMAP array.

expression of TNFα, we also observed an ascending trend, with a 3.3-fold increase in stage 4 versus the control, and as for CKD stages 3 and 4, the increases were 1.8-fold and 1.7-fold, respectively (Figure 6(b)).

Given the fact that various cytokines mediate the inflammatory response, the extent to which inflammation plays a role in raising the risk of MBDs in CKD remains unclear. Regarding the MBD molecules, we analysed the serum levels for OPG, OC, OPN, and FGF-23. All these biomarkers presented with an upward trend of expression, correlated with disease severity. In CKD stage 4, the circulatory levels showed the most significant differences compared to the control, as follows: for OPG, a 3.14-fold increase; for OC, a 4.6-fold increase; for OPN, a 7-fold increase; and for FGF-23, a 17-fold increase. Our results suggest that the serum levels of the above-mentioned molecules start to increase progressively, even from the CKD early stage 2, as depicted in Figures 6(c)–6(f).

Since all the analysed biomarkers expressed the highest concentrations in the most advanced stage of the disease, and given that the circulatory trend increases as the disease evolves, we considered it necessary to further analyse the possible correlations between these molecules that had a potential impact on CKD pathogenesis.

3.5. Correlations between Orchestrators of Inflammatory Response and Biomarkers of Mineral and Bone Disorders in CKD

3.5.1. The Trend for Biomarker Expression Was Modified Depending on the CKD Stage. The pathophysiologic interplay between mediators of inflammation and the molecules involved in MBDs was further analysed to establish potential significant correlations at each stage of renal disease. By applying the Chi-square test for trends, it was found that each CKD stage had its own unique biomarker signature.

In CKD stage 4, we found a strong positive correlation between Dkk-1 and calcitriol and a negative correlation between Dkk-1 and IL-6, OPG, OC, OPN, and FGF-23 ($p < 0.001$, Chi-square test for trends). Renal function (eGFR) was positively correlated with Dkk-1 in CKD stage 4 ($p < 0.001$).

Yeremenko et al. also observed an inverse correlation between Dkk-1 and IL-6 in a study on inflamed arthritic joints, potentially reflected by the differential regulation of Dkk-1 production by TNFα and IL-6 [41]. Besides, it was suggested that there were other recognised signalling pathways that Dkk-1 utilises other than the well-known canonical Wnt pathway [42]. Another study highlighted that the

production of proinflammatory cytokines IL-4 and IL-10 was notably reduced by Dkk-1 inhibitor treatment, suggesting that Dkk-1 utilises the MAPK and mTOR signalling pathway components to induce type 2 cell-mediated immune responses or inflammation [43]. In a study conducted by Malysheva et al., it was shown that proinflammatory cytokine IL-6 repressed the activation of the Wnt signalling pathway in human synoviocyte cells, and together with TNFα and Dkk-1, it inhibited the activation of the Wnt response [44].

It was also found that calcitriol distinctly regulated two genes encoding the extracellular Wnt inhibitors Dkk-1 and Dkk-4 via an indirect transcriptional mechanism. Thus, calcitriol increases the expression of Dkk-1 RNA and protein, acting as a tumour suppressor in human colon cancer cells harbouring endogenous mutations in the Wnt–β-catenin pathway [45].

Moreover, in CKD stage 4, the serum calcitriol concentrations were significantly correlated with proinflammatory cytokine TNFα ($p < 0.01$, Chi-square test) and the MBD markers OC, OPN, and FGF-23.

Our findings support the hypothesis that Dkk-1 could be a useful biomarker for CKD severity, together with calcitriol, both expressing the lowest levels in CKD stage 4.

According to recent studies, serum Dkk-1 levels were lower in CKD patients, displaying different kinetics depending on the disease stage [31].

We also obtained significant correlations between Dkk-1 and calcitriol in CKD stages 3 and 2 and with several proinflammatory and MBD markers, as follows: Dkk-1 and OPG, OPN, and FGF-23 ($p < 0.001$, Chi-square test for trends) in CKD stages 3 and 2; calcitriol and TNFα ($p < 0.01$) in CKD stage 3; and Dkk-1 and OPG and FGF-23 ($p < 0.001$) in CKD stage 2.

According to our results, Dkk-1, calcitriol, mediators of inflammation, and MBD markers showed significant interactions, also being correlated with the severity of CKD. How the relative balance between Dkk-1 and other cytokines determines Wnt signalling and the pattern of inflammation in CKD's different stages needs to be further investigated.

3.5.2. Strong Correlations between Dkk-1 and Calcitriol, Inflammatory Cytokines, and Renal Function in the CKD Patient Groups. The investigation of correlations in the CKD patient groups was examined by applying the χ^2 test (χ^2, p) for serum levels of all the above-mentioned markers, and strong correlations were found between Dkk-1 and calcitriol ($\chi^2 = 21.4$, $p < 0.001$). Furthermore, Dkk-1 was also strongly correlated with the mediators of inflammation IL-6 ($\chi^2 = 13.7$, $p < 0.001$) and TNFα ($\chi^2 = 10.4$, $p = 0.001$) and with the MBD biomarker FGF-23 ($\chi^2 = 10$, $p = 0.001$).

Calcitriol expression in the CKD patient groups was correlated with IL-6 ($\chi^2 = 4.4$, $p < 0.05$) and FGF-23 ($\chi^2 = 5.5$, $p = 0.01$). Regarding renal function, we found a strong correlation between eGFR and Dkk-1 ($\chi^2 = 8.48$, $p < 0.01$) and calcitriol ($\chi^2 = 8.36$, $p < 0.01$), indicating the increased potential for these two molecules in terms of assessing the severity of the disease.

In order to reveal the significant biomarker correlations between the CKD stages, we performed Spearman correlation tests (r, p value). In advanced CKD stage 4, we obtained significant correlations, as follows: TNFα and Dkk-1 ($r = 0.50$, $p < 0.05$), OPG ($r = 0.58$, $p < 0.05$), and OPN ($r = 0.66$, $p = 0.001$). The MBD biomarkers OPG and OPN were also correlated ($r = 0.51$, $p < 0.05$).

Other studies also supported the interactions between the key players of bone metabolism, Dkk-1 and OPG, in modulating the Wnt signalling pathway by balancing out bone absorption and reconstruction. TNF-α, a key inducer of Dkk-1, alongside OPG emerged as independent predictors of osteoarthritis severity. TNF-α, Dkk-1, and OPG were considered as valuable biomarkers in predicting the severity of the disease. The study also supported inflammation-induced Dkk-1 and OPG in osteoarthritis pathogenesis [46].

In CKD stage 3, correlations between the proinflammatory biomarkers TNFα and OPG ($r = 0.6$, $p = 0.001$) and FGF-23 ($r = 0.57$, $p < 0.01$) are highlighted. In CKD early stage 2, we found a strong negative correlation between Dkk-1 and FGF-23 ($r = -0.84$, $p < 0.001$); moderate correlations were also observed between calcitriol and IL-6 ($r = 0.53$, $p < 0.05$), TNFα ($r = 0.58$, $p < 0.05$), OPG ($r = 0.71$, $p < 0.05$), and FGF-23 ($r = 0.52$, $p < 0.05$). The mediators of inflammation, IL-6 and TNFα, were also moderately correlated ($r = 0.58$, $p < 0.05$), and a moderate correlation was found between IL-6 and OPG ($r = 0.61$, $p = 0.01$).

In CKD, a complex network between Dkk-1, calcitriol, mediators of inflammation, and MBD markers exists, but the level at which it can affect the course of the disease remains in question.

3.5.3. Significant Differences between Dkk-1, Calcitriol, Mineral Disorders, Inflammatory Markers, and Renal Function, Depending on CKD Stages. By applying the Kruskal-Wallis one-way analysis of variance, we obtained significant differences in the circulating expression of Dkk-1, calcitriol, and eGFR in CKD patients ($p < 0.0001$). The post hoc analysis showed that levels of Dkk-1, calcitriol, and eGFR were significantly different between CKD stage 4 and stage 3, CKD stages 4 and 2, and CKD stages 3 and 2, respectively ($p < 0.0001$), highlighting the potential of these two markers in evaluating the severity of the disease.

Significant differences in IL-6 were observed in CKD patients ($p < 0.0001$). Bonferroni's multiple comparison test showed that IL-6 was significantly different between CKD stage 4 and stage 3, CKD stages 4 and 2 ($p < 0.0001$), and CKD stages 3 and 2 ($p < 0.05$). TNFα showed a significant variance in CKD patients ($p < 0.01$), and the differences between the stages were as follows: CKD stage 4 and stage 3 and CKD stages 4 and 2 ($p < 0.05$), according to our post hoc analysis.

FGF-23 and OC presented with significant differences in the CKD group ($p < 0.0001$), and the comparisons between stages were only significant between CKD stage 4 and stage 3 and CKD stages 4 and 2 ($p < 0.0001$).

According to our results, we can conclude that a crosstalk between Dkk-1, calcitriol, mineral disorders, inflammation,

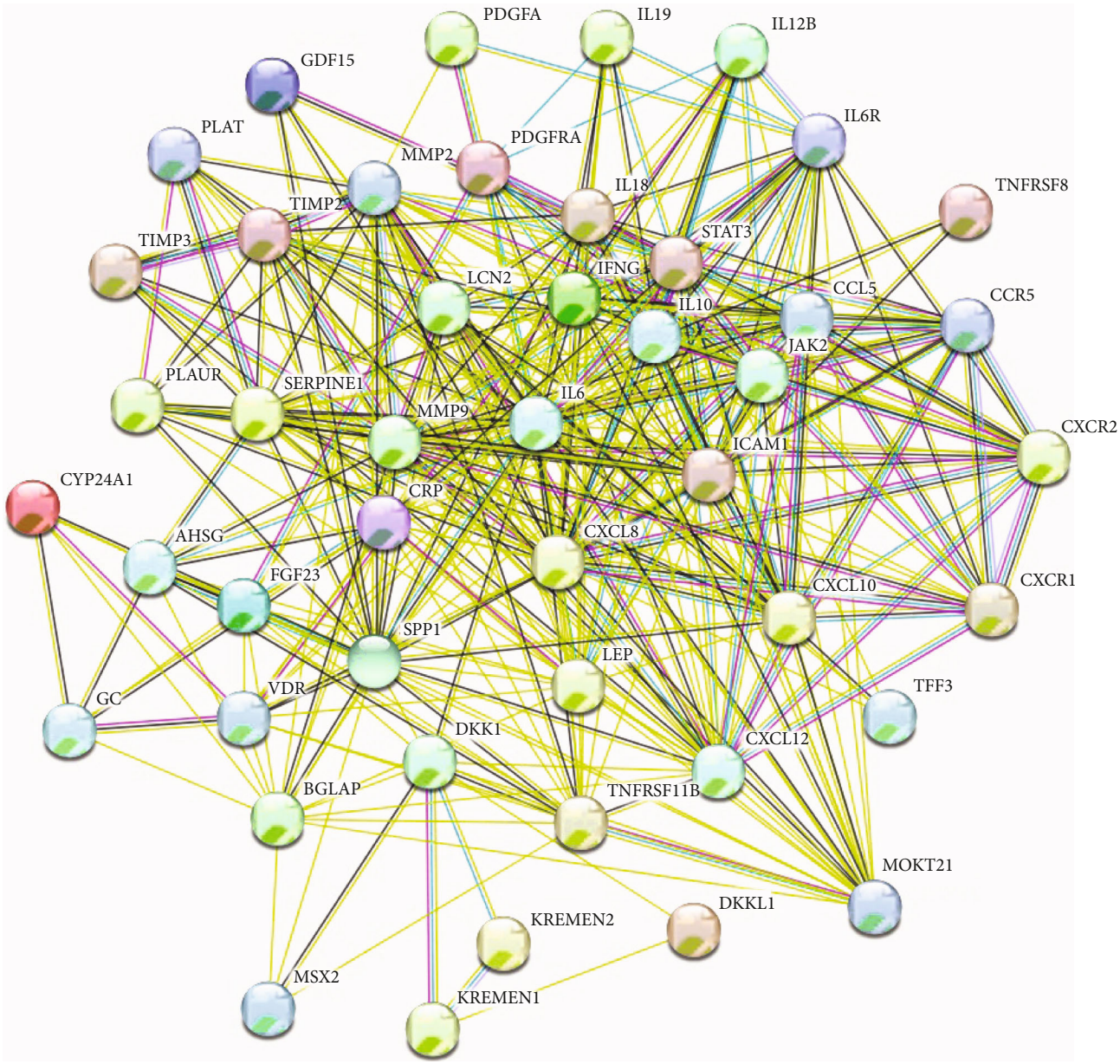

Figure 7: Functional interaction between different molecules involved in inflammation and MBDs in CKD. The coloured nodes are represented by query proteins and the first shell of interactors. Edges represent protein-protein functional associations, assigned with different colour codes, as follows: a blue edge indicates known interactions from curated databases, a pink edge indicates known interactions that have been experimentally determined, a green edge indicates predicted interactions in the gene neighbourhood, a red edge indicates predicted interactions for gene fusions, a blue-ink edge indicates predicted interactions for gene cooccurrences, a light-green edge indicates other interactions derived from text mining, and a black edge indicates gene coexpression derived from other databases. Abbreviations: CYP24A1: calcitriol, 1,25-dihydroxyvitamin D_3, and $1,25OH_2D_3$; SPP1: osteopontin, OPN; TNFRSF11B: osteoprotegerin, OPG; BGLAP: osteocalcin, OC; CXCL8: IL-8, interleukin-8; GC: vitamin D binding protein, DBP; VDR: vitamin D receptor.

and renal function is present in CKD, thus influencing CKD pathophysiology. Inflammation, the hallmark feature of chronic diseases, seems to be a common mediator for both kidney function and subsidiary MBDs. Because of its insidious nature, CKD silently evolves alongside other chronic conditions, exhibiting different biomarker patterns depending on disease severity.

3.6. Functional Interplay between Markers of Inflammation and Mineral Bone Disorders in CKD. Considering the relevant proteins revealed by dot-blot immunodetection screening, the functional interactions between the molecules involved in shaping the different patterns of CKD have been put together by employing the STRING databases. The interactions include functional associations between multiple molecules stemming from computational prediction, knowledge transfer between organisms, and interactions derived from other databases. Stronger evidence for an association is represented by a thicker network edge, as depicted in Figure 7.

Since CKD commonly arises alongside other comorbidities (such as hypertension, diabetes, and CVD) and the diagnosis of isolated CKD represents the exception rather than the rule, an integrative patient assessment is the best clinical approach [47]. Detailed characterisation of kidney disease is needed to better understand the molecular relationships underlying the pathophysiology of disease and to design CKD biomarker patterns characteristic of the various CKD stages, thus moving towards personalised care for each individual patient.

A potential limitation of our study is its cross-sectional design, given the relatively small number of patients included in our study. Therefore, further intense research is necessary to completely decipher the underlying mechanisms behind the connections between the analysed molecules in order to better characterise the cytokine patterns in CKD.

4. Conclusions

As highlighted in our study, a functional interplay occurs between markers of inflammation and MBDs in CKD depending on disease severity. In spite of the advances in CKD pathophysiology, there is an emerging need for novel biomarkers to better characterise the different patterns of nephropathy at each CKD stage. Out of all the analysed molecules, Dkk-1 and calcitriol were found to significantly correlate with CKD clinical staging, exhibiting the lowest levels in CKD stage 4. Since inflammation has emerged at the core of the pathophysiology of CKD, our results revealed significant correlations between Dkk-1 and calcitriol and proinflammatory cytokines, starting with the early CKD stages. The MBD biomarkers OPG, OPN, OC, and FGF-23 were significantly correlated with Dkk-1 and calcitriol, as well as with the mediators of inflammation IL-6 and TNFα. In view of these findings, Dkk-1 and calcitriol could be considered as potential useful biomarkers for CKD severity. Nevertheless, further studies are needed to clearly unravel the complex networking between Dkk-1, calcitriol, the mediators of inflammation, and MBD markers to design promising biomarker patterns for CKD, starting with its early stages.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

All authors contributed equally to this work.

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References

[1] T. Isakova, T. L. Nickolas, M. Denburg et al., "KDOQI US commentary on the 2017 KDIGO clinical practice guideline update for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease–mineral and bone disorder (CKD-MBD)," *American Journal of Kidney Diseases*, vol. 70, no. 6, pp. 737–751, 2017.

[2] B. L. Neuen, S. J. Chadban, A. R. Demaio, D. W. Johnson, and V. Perkovic, "Chronic kidney disease and the global NCDs agenda," *BMJ Global Health*, vol. 2, no. 2, article e000380, 2017.

[3] H. Wang, M. Naghavi, C. Allen et al., "Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015," *The Lancet*, vol. 388, no. 10053, pp. 1459–1544, 2016.

[4] N. R. Hill, S. T. Fatoba, J. L. Oke et al., "Global prevalence of chronic kidney disease – a systematic review and meta-analysis," *PLoS One*, vol. 11, no. 7, article e0158765, 2016.

[5] J. Voelkl, F. Lang, K. U. Eckardt et al., "Signaling pathways involved in vascular smooth muscle cell calcification during hyperphosphatemia," *Cellular and Molecular Life Sciences*, vol. 76, no. 11, pp. 2077–2091, 2019.

[6] K. A. Hruska, T. Sugatani, O. Agapova, and Y. Fang, "The chronic kidney disease - mineral bone disorder (CKD-MBD): advances in pathophysiology," *Bone*, vol. 100, pp. 80–86, 2017.

[7] C. Mambet, L. Necula, S. Mihai et al., "Increased Dkk-1 plasma levels may discriminate disease subtypes in myeloproliferative neoplasms," *Journal of Cellular and Molecular Medicine*, vol. 22, no. 8, pp. 4005–4011, 2018.

[8] K. C. Lu, C. C. Wu, J. F. Yen, and W. C. Liu, "Vascular calcification and renal bone disorders," *The Scientific World Journal*, vol. 2014, Article ID 637065, 20 pages, 2014.

[9] G. Jean, J. Souberbielle, and C. Chazot, "Vitamin D in chronic kidney disease and dialysis patients," *Nutrients*, vol. 9, no. 4, p. 328, 2017.

[10] Y. Nishikawa, R. Nakayama, S. Obika, E. Ohsaki, K. Ueda, and T. Honda, "Inhibition of LINE-1 retrotransposition by capsaicin," *International Journal of Molecular Sciences*, vol. 19, no. 10, p. 3243, 2018.

[11] R. L. Amdur, H. I. Feldman, J. Gupta et al., "Inflammation and progression of CKD: the CRIC Study," *Clinical Journal of the American Society of Nephrology*, vol. 11, no. 9, pp. 1546–1556, 2016.

[12] S. Salimi, M. D. Shardell, S. L. Seliger, S. Bandinelli, J. M. Guralnik, and L. Ferrucci, "Inflammation and trajectory of renal function in community-dwelling older adults," *Journal of the American Geriatrics Society*, vol. 66, no. 4, pp. 804–811, 2018.

[13] S. Mihai, E. Codrici, I. D. Popescu et al., "Proteomic biomarkers panel: new insights in chronic kidney disease," *Disease Markers*, vol. 2016, Article ID 3185232, 11 pages, 2016.

[14] W. Chen and D. A. Bushinsky, "Chronic kidney disease: KDIGO CKD-MBD guideline update: evolution in the face of

uncertainty," *Nature Reviews Nephrology*, vol. 13, no. 10, pp. 600–602, 2017.

[15] C. Tanase, R. Albulescu, E. Codrici et al., "Decreased expression of APAF-1 and increased expression of cathepsin B in invasive pituitary adenoma," *OncoTargets and Therapy*, vol. 8, pp. 81–90, 2015.

[16] M. H. Hanna, A. Dalla Gassa, G. Mayer et al., "The nephrologist of tomorrow: towards a kidney-omic future," *Pediatric Nephrology*, vol. 32, no. 3, pp. 393–404, 2017.

[17] S. Mihai, E. Codrici, I. D. Popescu et al., "Inflammation-related mechanisms in chronic kidney disease prediction, progression, and outcome," *Journal of Immunology Research*, vol. 2018, Article ID 2180373, 16 pages, 2018.

[18] L. Dai, E. Golembiewska, B. Lindholm, and P. Stenvinkel, "End-stage renal disease, inflammation and cardiovascular outcomes," *Contributions to Nephrology*, vol. 191, pp. 32–43, 2017.

[19] H. Su, C. T. Lei, and C. Zhang, "Interleukin-6 signaling pathway and its role in kidney disease: an update," *Frontiers in Immunology*, vol. 8, p. 405, 2017.

[20] K. Yong, E. M. Ooi, G. Dogra et al., "Elevated interleukin-12 and interleukin-18 in chronic kidney disease are not associated with arterial stiffness," *Cytokine*, vol. 64, no. 1, pp. 39–42, 2013.

[21] M. Del Rosso, F. Margheri, S. Serrati, A. Chilla, A. Laurenzana, and G. Fibbi, "The urokinase receptor system, a key regulator at the intersection between inflammation, immunity, and coagulation," *Current Pharmaceutical Design*, vol. 17, no. 19, pp. 1924–1943, 2011.

[22] T. Liu, L. Zhang, D. Joo, and S. C. Sun, "NF-κB signaling in inflammation," *Signal Transduction and Targeted Therapy*, vol. 2, no. 1, 2017.

[23] H. Zhang and S. C. Sun, "NF-κB in inflammation and renal diseases," *Cell & Bioscience*, vol. 5, no. 1, 2015.

[24] G. Lui, C. K. Wong, M. Ip et al., "HMGB1/RAGE signaling and pro-inflammatory cytokine responses in non-HIV adults with active pulmonary tuberculosis," *PLoS One*, vol. 11, no. 7, article e0159132, 2016.

[25] D. V. Barreto, A. Lenglet, S. Liabeuf et al., "Prognostic implication of plasma osteopontin levels in patients with chronic kidney disease," *Nephron Clinical Practice*, vol. 117, no. 4, pp. c363–c372, 2011.

[26] P. Evenepoel, P. D'Haese, and V. Brandenburg, "Sclerostin and DKK1: new players in renal bone and vascular disease," *Kidney International*, vol. 88, no. 2, pp. 235–240, 2015.

[27] B. Thompson and D. A. Towler, "Arterial calcification and bone physiology: role of the bone-vascular axis," *Nature Reviews Endocrinology*, vol. 8, no. 9, pp. 529–543, 2012.

[28] A. Gay and D. A. Towler, "Wnt signaling in cardiovascular disease: opportunities and challenges," *Current Opinion in Lipidology*, vol. 28, no. 5, pp. 387–396, 2017.

[29] D. Wo, J. Peng, D. N. Ren et al., "Opposing roles of Wnt inhibitors IGFBP-4 and Dkk1 in cardiac ischemia by differential targeting of LRP5/6 and β-catenin," *Circulation*, vol. 134, no. 24, pp. 1991–2007, 2016.

[30] K. Hamada-Ode, Y. Taniguchi, Y. Shimamura, S. Fujimoto, and Y. Terada, "Serum dickkopf-related protein 1 and sclerostin may predict the progression of chronic kidney disease in Japanese patients," *Nephrology, Dialysis, Transplantation*, vol. 34, no. 8, pp. 1426-1427, 2019.

[31] G. J. Behets, L. Viaene, B. Meijers et al., "Circulating levels of sclerostin but not DKK1 associate with laboratory parameters of CKD-MBD," *PLoS One*, vol. 12, no. 5, article e0176411, 2017.

[32] The PLOS ONE Staff, "Correction: relationship of Dickkopf1 (DKK1) with cardiovascular disease and bone metabolism in Caucasian type 2 diabetes mellitus," *PLoS One*, vol. 10, no. 1, article e0117687, 2015.

[33] S. U. Kim, J. H. Park, H. S. Kim et al., "Serum Dickkopf-1 as a biomarker for the diagnosis of hepatocellular carcinoma," *Yonsei Medical Journal*, vol. 56, no. 5, pp. 1296–1306, 2015, Epub 2015/08/11.

[34] M. Rogers, C. Goettsch, and E. Aikawa, "Medial and intimal calcification in chronic kidney disease: stressing the contributions," *Journal of the American Heart Association*, vol. 2, no. 5, article e000481, 2013.

[35] J. Jang, Y. Jung, Y. Kim, E. H. Jho, and Y. Yoon, "LPS-induced inflammatory response is suppressed by Wnt inhibitors, Dickkopf-1 and LGK974," *Scientific Reports*, vol. 7, no. 1, article 41612, 2017.

[36] C. Liu and H. Li, "Correlation of the severity of chronic kidney disease with serum inflammation, osteoporosis and vitamin D deficiency," *Experimental and Therapeutic Medicine*, vol. 17, no. 1, pp. 368–372, 2018.

[37] C. L. Lu, D. F. Yeih, Y. C. Hou et al., "The emerging role of nutritional vitamin D in secondary hyperparathyroidism in CKD," *Nutrients*, vol. 10, no. 12, p. 1890, 2018.

[38] A. Gil, J. Plaza-Diaz, and M. D. Mesa, "Vitamin D: classic and novel actions," *Annals of Nutrition & Metabolism*, vol. 72, no. 2, pp. 87–95, 2018.

[39] D. Mattinzoli, M. Ikehata, K. Tsugawa et al., "FGF23 and Fetuin-A interaction and mesenchymal osteogenic transformation," *International Journal of Molecular Sciences*, vol. 20, no. 4, p. 915, 2019.

[40] H. Haddad Kashani, E. Seyed Hosseini, H. Nikzad et al., "The effects of vitamin D supplementation on signaling pathway of inflammation and oxidative stress in diabetic hemodialysis: a randomized, double-blind, *placebo-controlled trial*," *Frontiers in Pharmacology*, vol. 9, p. 50, 2018.

[41] N. Yeremenko, K. Zwerina, G. Rigter et al., "Brief Report: Tumor necrosis factor and interleukin-6 differentially regulate Dkk-1 in the inflamed arthritic joint," *Arthritis & Rheumatology*, vol. 67, no. 8, pp. 2071–2075, 2015.

[42] T. Fukuda, S. Kokabu, S. Ohte et al., "Canonical Wnts and BMPs cooperatively induce osteoblastic differentiation through a GSK3β-dependent and β-catenin-independent mechanism," *Differentiation*, vol. 80, no. 1, pp. 46–52, 2010.

[43] W. J. Chae, A. K. Ehrlich, P. Y. Chan et al., "The Wnt antagonist Dickkopf-1 promotes pathological type 2 cell-mediated inflammation," *Immunity*, vol. 44, no. 2, pp. 246–258, 2016.

[44] K. Malysheva, K. de Rooij, C. W. G. M. Löwik et al., "Interleukin 6/Wnt interactions in rheumatoid arthritis: interleukin 6 inhibits Wnt signaling in synovial fibroblasts and osteoblasts," *Croatian Medical Journal*, vol. 57, no. 2, pp. 89–98, 2016.

[45] N. Pendas-Franco, O. Aguilera, F. Pereira, J. M. Gonzalez-Sancho, and A. Munoz, "Vitamin D and Wnt/beta-catenin pathway in colon cancer: role and regulation of DICKKOPF genes," *Anticancer Research*, vol. 28, no. 5A, pp. 2613–2623, 2008.

[46] S. Min, C. Wang, W. Lu et al., "Serum levels of the bone turnover markers dickkopf-1, osteoprotegerin, and TNF-α in knee osteoarthritis patients," *Clinical Rheumatology*, vol. 36, no. 10, pp. 2351–2358, 2017.

[47] S. D. Fraser and T. Blakeman, "Chronic kidney disease: identification and management in primary care," *Pragmatic and Observational Research*, vol. 7, pp. 21–32, 2016.

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Research Article

Ratio of Early Mitral Inflow Velocity to the Global Diastolic Strain Rate and Global Left Ventricular Longitudinal Systolic Strain Predict Overall Mortality and Major Adverse Cardiovascular Events in Hemodialysis Patients

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Background. The ratio of early mitral inflow velocity to the global diastolic strain rate (E/E'sr) and global longitudinal systolic strain (GLS) of the left ventricle (LV) are emerging indices of diastolic and systolic functions, respectively, for the LV. Their prognostic significance in the prediction of mortality and cardiovascular (CV) outcomes remains underexplored in hemodialysis (HD) patients. *Methods*. This prospective study included 190 maintenance HD patients. The E/E'sr ratio and GLS were assessed using two-dimensional speckle tracking echocardiography. The clinical outcomes included overall mortality, CV mortality, and major adverse cardiovascular events (MACE). The associations between the E/E'sr ratio, GLS, and clinical outcomes were evaluated using multivariate Cox regression analysis. The incremental values of the E/E'sr ratio and GLS in outcome prediction were assessed by χ^2 changes in Cox models. *Results*. Over a median follow-up period of 3.7 years, there were 35 overall deaths, 16 CV deaths, and 45 MACE. Impaired diastolic function with a higher E/E'sr ratio was associated with overall mortality (HR, 1.484; 95% CI, 1.201−1.834; $p < 0.001$), CV mortality (HR, 1.584; 95% CI, 1.058–2.371; $p = 0.025$), and MACE (HR, 1.205; 95% CI, 1.040−1.397; $p = 0.013$) in multivariate adjusted Cox analysis. Worsening GLS was associated with overall mortality (HR, 1.276; 95% CI, 1.101−1.480; $p = 0.001$), CV mortality (HR, 1.513; 95% CI, 1.088–2.104; $p = 0.014$), and MACE (HR, 1.214; 95% CI, 1.103−1.337; $p < 0.001$). The E/E'sr ratio and GLS had better outcome prediction than the E to early diastolic mitral annular velocity (E/E') ratio and left ventricular ejection fraction (LVEF). Moreover, adding the E/E'sr ratio and GLS to Cox models containing relevant clinical and conventional echocardiographic parameters improved the prediction of overall mortality ($p < 0.001$), CV mortality ($p < 0.001$), and MACE ($p < 0.001$). *Conclusion*. The E/E'sr ratio and GLS, as emerging indices of LV diastolic and systolic functions, significantly predict mortality and CV outcomes and outperform conventional echocardiographic parameters in outcome prediction in HD patients.

1. Introduction

Cardiovascular (CV) disease is the leading cause of mortality in patients undergoing hemodialysis (HD) [1]. Higher prevalence of traditional risk factors and functional abnormalities of the heart may contribute to this high CV risk in end-stage renal disease (ESRD) [2, 3]. Pressure and volume overload could cause such cardiac abnormalities [4, 5]. Two-dimensional (2D) speckle tracking echocardiography (STE) allows for angle-independent quantification of myocardial deformation to more accurately reflect systolic and diastolic performances of all myocardial segments [6, 7].

2D STE can assess the left ventricular (LV) early global diastolic strain rate (E'sr). Furthermore, the early mitral inflow velocity (E) to E'sr ratio has been reported to be an emerging index of LV diastolic function [8, 9] and is strongly correlated with invasively measured LV filling pressure [10–12]. The E/E'sr ratio is associated with unfavorable outcomes among patients with acute myocardial infarction [13] and systolic heart failure [14]. Global LV longitudinal systolic strain (GLS) has been recognized as a proper indicator of LV systolic function [7, 15]. Less negative GLS is associated with an increased risk of death in patients undergoing dialysis [16, 17]. However, the associations between the E/E'sr ratio and the risk of mortality and CV outcomes have never been investigated in chronic HD patients. Therefore, this study is aimed at examining the prognostic significance of the E/E'sr ratio and GLS in the prediction of overall mortality, CV mortality, and major adverse cardiovascular events (MACE) in maintenance HD patients. We further explored whether the emerging indices of LV diastolic and systolic functions outperformed the conventional echocardiographic parameters in the prediction of mortality and CV outcomes.

2. Materials and Methods

2.1. Study Patients. The inclusion criterion of the present study was patients with maintenance hemodialysis (HD) > 3 months at the outpatient HD unit. This study enrolled 219 maintenance HD patients at a regional hospital in Taiwan from March to October 2014. Patients with refusal of examinations ($n = 18$), lack of STE measurements ($n = 7$), and atrial fibrillation ($n = 4$) were excluded. No patient was excluded because of poor echogenicity. Overall, 190 study patients were included (Figure 1). The study adhered to the Declaration of Helsinki and was approved by the Institutional Review Board of Kaohsiung Medical University Hospital, and all participants provided their written informed consent.

2.2. Echocardiographic Measurements. Patients received echocardiographic measurements in the left decubitus position by one well-experienced cardiologist, using a Vivid 7 system (GE Vingmed Ultrasound AS, Horten, Norway). The cardiologist was blind to patients' clinical information. Early diastolic velocities (E') of lateral and septal mitral annuli were averaged using Doppler tissue imaging to calculate the E/E' ratio. The LV ejection fraction (LVEF) and LV mass were calculated using the biplane Simpson's and Devereux's methods, respectively [6]. The LV mass index (LVMI) was calculated as LV mass divided by the body surface area. Left atrial volume was calculated using the biplane area-length method. The left atrial volume index (LAVI) was defined as left atrial volume divided by the body surface area. Relative wall thickness was calculated by (2 × posterior wall thickness in diastole)/LV diastolic diameter. All volumetric measurements and analyses were performed in accordance with EAE/ASE recommendations [6].

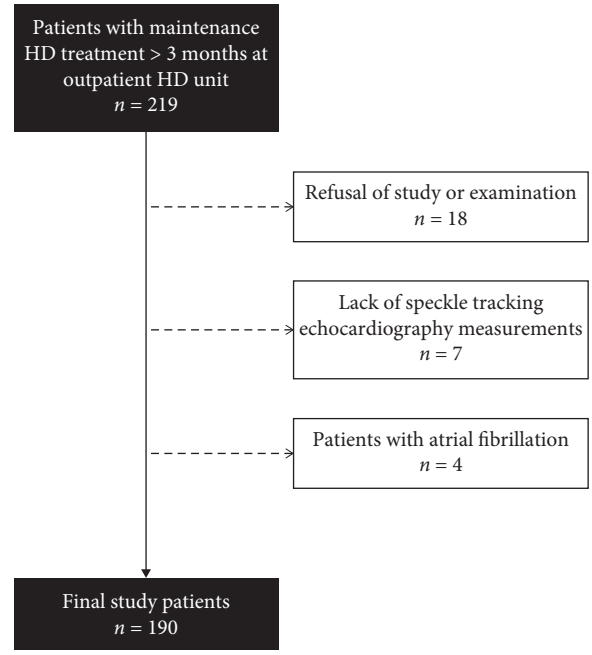

Figure 1: Flowchart of participants analyzed in this study.

LV apical two- and four-chamber and long-axis views were obtained. The endocardial border was defined manually, and epicardial surface tracing was automatically performed by the system to create a region of interest [18]. The LV chamber was divided into six segments, with their strain and strain rate curves being analyzed. The peak segmental longitudinal systolic strain and early diastolic strain rates were determined from these curves (Figure 2). The E'sr and GLS were assessed and averaged in 18 LV segments from the three standard apical views (four-, two-, and three-chamber views). All the 18 speckle tracking segments were kept into the analysis in all patients. LV dimensions, LVEF, LAVI, LVMI, E'sr, and GLS were measured from the index beat [9, 19, 20]. A single beat was analyzed each time, and the values from three cardiac cycles were average to obtain each index. All STE data were recorded and analyzed offline using EchoPAC version 08.

2.3. Assessment of the Ankle-Brachial Index (ABI) and Brachial-Ankle Pulse Wave Velocity (baPWV). ABI and baPWV were measured 10–30 minutes before the HD session using an ABI-form analyzer (Colin VP1000, Komaki, Japan) which simultaneously measured blood pressure in both arms and ankles. ABI was calculated as systolic blood pressure of the ankles divided by systolic blood pressure of the arms. The baPWV was automatically calculated as the

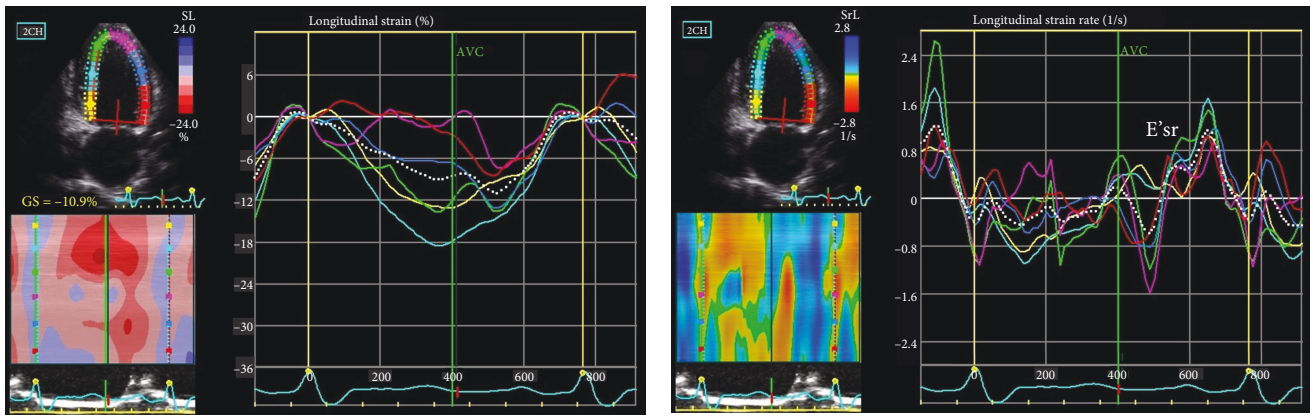

Figure 2: A representative example of measurements of global longitudinal strain (a) and the early global diastolic strain rate (b) from the curves of longitudinal strain and the strain rate of six segments of the left ventricle in the apical two-chamber view. AVC: aortic valve closure; E'sr: early global diastolic strain rate.

transmission distance divided by the transmission time. The ABI is a simple and noninvasive test for establishing the diagnosis of peripheral artery disease (PAD) and a marker of generalized atherosclerosis, which are prevalent among HD patients and associated with worse clinical outcomes [21]. PAD was defined as an ABI < 0.95 [22, 23].

2.4. Evaluation of Aortic Arch Calcification (AoAC) and the Cardiothoracic Ratio by Chest X-Rays. An experienced radiologist blind to the patients' clinical information reviewed their chest X-rays and assessed AoAC using the scale proposed by Ogawa et al. [24]. The aortic arch was divided into 16 sections on the chest X-rays, and the number of sections with calcification was counted. The cardiothoracic ratio, assessed by the radiologist, was defined as the ratio of the transverse diameter of the cardiac shadow to the transverse diameter of the chest on the chest X-rays.

2.5. Demographic, Medical, and Laboratory Data. Demographic and medical data including age, gender, and comorbidities were obtained from patients' medical records and interviews. Laboratory tests were conducted using overnight fasting blood samples obtained within 1 month of enrollment. Information of the use of medications, including angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), β-blockers, and statins, was obtained from medical records.

2.6. Definition of Overall Mortality, CV Mortality, and MACE. All study patients' medical records and hospital courses were reviewed by two cardiologists to define the cause of death and MACE. CV mortality was defined as sudden cardiac death, fatal myocardial infarction, ventricular arrhythmia, fatal stroke, and heart failure. MACE was defined as follows: hospitalization for unstable angina, nonfatal myocardial infarction, sustained ventricular arrhythmia, hospitalization for congestive heart failure, transient ischemia attack or stroke, hospitalization for peripheral artery occlusive disease, and CV death [25]. If the patients experienced more than one MACE, only the first was analyzed. The model for MACE was censored at the development of MACE or the end of follow-up. All patients were followed until December 31, 2017, or the study endpoint (overall or CV mortality).

2.7. Statistical Analysis. All statistical analyses were carried out using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) for Windows. Data are expressed as percentages, mean ± standard deviation, or median (25^{th}–75^{th} percentile) for the dialysis vintage, triglycerides, high-sensitivity C-reactive protein (hs-CRP), and AoAC. The study patients were stratified into three groups according to tertiles of the E/E'sr ratio, with the 1^{st} tertile as the reference category. Multiple comparisons among the study groups were performed using one-way analysis of variance followed by the post hoc Bonferroni test. These study patients were also classified into two groups according to a preserved GLS (≤−16%) or impaired GLS (>−16%) [26, 27]. Differences between groups were analyzed using the chi-square test for categorical variables and the independent t-test for continuous variables. Cox proportional hazards analysis was performed to evaluate the associations between the E/E'sr ratio and GLS and development of overall mortality, CV mortality, or MACE. The adjusted covariates included age, sex, dialysis vintage, diabetes mellitus (DM), coronary artery disease, cerebrovascular disease, body mass index, systolic blood pressure, ABI < 0.95, baPWV, the cardiothoracic ratio, AoAC, albumin, triglycerides, total cholesterol, hemoglobin, creatinine, calcium-phosphorous product, hs-CRP, and the use of ACE inhibitors or ARBs, β-blockers, and statins. Survival curves for cumulative probability of overall mortality, CV mortality and MACE were illustrated using the Kaplan-Meier method and compared among mentioned groups of patients by the log-rank test. Direct comparisons between the E/E' ratio and the E/E'sr ratio, as well as GLS and LVEF, were performed using multivariate models and assessed by changes in χ^2. Incremental model performance of the E/E'sr ratio and GLS over clinical and echocardiographic variables was assessed by comparing the model χ^2 at each step. A p value<0.05 was considered statistically significant.

3. Results

A total of 190 study patients were included. The mean age was 60.7 ± 11.7 years, and there were 98 males and 92 females. The mean values of the E/E'sr ratio and GLS were 64.5 ± 25.5 cm and $-16.8 \pm 4.1\%$, respectively. The patients were stratified into three groups according to tertiles of the E/E'sr ratio (<50.4 cm, 50.4–67.9 cm, and >67.9 cm). Patients belonging to the 3rd tertile of the E/E'sr ratio had higher prevalence of DM and coronary artery disease, higher cardiothoracic ratio, higher AoAC, lower serum creatinine, higher prevalence of ACE inhibitors or ARB and β-blocker use, higher LAVI, higher LVMI, lower LVEF, higher E/E' ratio, higher E/A ratio, lower E' wave, lower E'sr, and less negative GLS compared to patients in the 1st tertile of the E/E'sr ratio (Table 1).

Table 2 shows the comparison of baseline characteristics between patients with preserved GLS (≤−16%) or impaired GLS (>−16%). Compared to patients with preserved GLS, those with impaired GLS had higher prevalence of DM, coronary artery disease, and cerebrovascular disease; higher systolic blood pressure; higher prevalence of ABI < 0.95; higher AoAC; higher hemoglobin; higher calcium-phosphorous product; higher LVMI; lower LVEF; lower E' wave; lower E'sr; and higher E/E'sr ratio.

As shown in Figure 3(a), the cumulative incidence rates of overall mortality, CV mortality, and MACE were highest among patients in the 3rd tertile of the E/E'sr ratio, intermediate among those in the 2nd tertile and lowest among those in the 1st tertile ($p < 0.001$ for the trend). The incidence rates of overall mortality ($p = 0.006$), CV mortality ($p = 0.024$), and MACE ($p < 0.001$) were higher among patients with impaired GLS in comparison with those with preserved GLS (Figure 3(b)).

3.1. Risk of Overall Mortality. Over a median follow-up of 3.7 years (interquartile range: 3.3-3.8 years), there were 35 (18.4%) deaths, including fatal CV events ($n = 16$), sepsis or septic shock ($n = 15$), gastrointestinal bleeding ($n = 2$), malignancy ($n = 1$), and liver failure ($n = 1$) among study patients. The Kaplan-Meier curves for the cumulative probability of overall mortality according to tertiles of the E/E'sr ratio (Figure 4(a)) show substantially higher probability of overall mortality among patients in the 3rd tertile of the E/E'sr ratio compared to patients in the 1st or 2nd tertile of the E/E'sr ratio ($p < 0.001$ by the log-rank test). As shown in Figure 5(a), patients with impaired GLS had higher probability of overall mortality compared to those with preserved GLS ($p = 0.005$ by the log-rank test).

Table 3 displays the hazard ratios (HR) of the E/E'sr ratio and GLS for overall mortality with and without adjustment for demographic, clinical, biochemical factors. A high E/E'sr ratio (per 10 cm) was significantly associated with overall mortality in the unadjusted model (HR, 1.191; 95% confidence interval (CI), 1.090–1.301; $p < 0.001$), in the age- and sex-adjusted model (HR, 1.185; 95% CI, 1.074–1.308; $p = 0.001$), and in the multivariable model adjusted for age, sex, dialysis vintage, DM, coronary artery disease, cerebrovascular disease, BMI, systolic blood pressure, ABI < 0.95, baPWV, cardiothoracic radio, AoAC, albumin, triglycerides, total cholesterol, hemoglobin, creatinine, calcium-phosphorous product, and hs-CRP. This association holds significant (HR, 1.484; 95% CI, 1.201–1.834; $p < 0.001$) after being further adjusted for medication use of ACE inhibitors or ARBs, β-blockers, and statins.

Furthermore, GLS (per 1%) was significantly associated with overall mortality in the unadjusted model (HR, 1.127; 95% CI, 1.043–1.219; $p = 0.003$), in the age- and sex-adjusted model (HR, 1.139; 95% CI, 1.046–1.239; $p = 0.003$), and in the full multivariable adjusted model (HR, 1.276; 95% CI, 1.101–1.480; $p = 0.001$).

3.2. Risk of CV Mortality. Sixteen (8.4%) CV deaths were recorded during the follow-up period, including sudden cardiac death ($n = 8$), myocardial infarction ($n = 4$), ventricular arrhythmia ($n = 1$), fatal stroke ($n = 2$), and heart failure ($n = 1$). The Kaplan-Meier curves (Figure 4(b)) show higher cumulative probability of CV mortality among patients in the 3rd tertile of the E/E'sr ratio compared to patients in the 1st or 2nd tertile of the E/E'sr ratio ($p < 0.001$ by the log-rank test). Figure 5(b) shows that compared to patients with preserved GLS, those with impaired GLS had higher cumulative probability of CV mortality ($p = 0.017$ by the log-rank test).

As shown in Table 3, a high E/E'sr ratio (per 10 cm) was associated with CV mortality in the unadjusted model (HR, 1.271; 95% CI, 1.135–1.423; $p < 0.001$), in the age- and sex-adjusted model (HR, 1.261; 95% CI, 1.112–1.429; $p < 0.001$), and in the full multivariable adjusted model (HR, 1.584; 95% CI, 1.058–2.371; $p = 0.025$). GLS (per 1%) was associated with CV mortality in the unadjusted model (HR, 1.202; 95% CI, 1.075–1.343; $p = 0.001$), in the age- and sex-adjusted model (HR, 1.208; 95% CI, 1.072–1.360; $p = 0.002$), and in the full multivariable adjusted model (HR, 1.513; 95% CI, 1.088–2.104; $p = 0.014$).

3.3. Risk of MACE. Forty-five (23.7%) MACE were documented during the follow-up period, including hospitalization for heart failure ($n = 5$), coronary artery disease ($n = 12$), ventricular arrhythmia ($n = 3$), stroke ($n = 3$), peripheral artery disease ($n = 6$), and CV deaths ($n = 16$). As shown in Figures 4(c) and 5(c), patients in the 3rd tertile of the E/E'sr ratio had higher cumulative probability of MACE compared to patients in the 1st or 2nd tertile of the E/E'sr ratio ($p < 0.001$ by the log-rank test) and higher cumulative probability of MACE among patients with impaired GLS compared to those with preserved GLS over the follow-up period ($p < 0.001$ by the log-rank test).

Table 3 shows that the high E/E'sr ratio (per 10 cm) was associated with MACE in the unadjusted model (HR, 1.188; 95% CI, 1.101–1.282; $p < 0.001$) and in the full multivariable adjusted model (HR, 1.205; 95% CI, 1.040–1.397; $p = 0.013$). GLS (per 1%) was associated with MACE in the unadjusted model (HR, 1.174; 95% CI, 1.100–1.254; $p < 0.001$) and in the full multivariable adjusted model (HR, 1.214; 95% CI, 1.103–1.337; $p < 0.001$).

3.4. Comparison of the E/E' Ratio and the E/E'sr Ratio to Overall Mortality, CV Mortality, and MACE. As shown in

Table 1: Comparison of baseline characteristics among patients according to tertiles of the E/E'sr ratio.

Characteristics	1st tertile of E/E'sr ratio (<50.4 cm) ($n = 63$)	2nd tertile of E/E'sr ratio (50.4–67.9 cm) ($n = 63$)	3rd tertile of E/E'sr ratio (>67.9 cm) ($n = 64$)	p
Age (year)	58.6 ± 10.6	60.6 ± 13.3	63.2 ± 11.0	0.083
Male gender (%)	49.2	47.6	57.8	0.465
Dialysis vintage (year)	7.6 (3.7–12.5)	6.6 (2.9–13.0)	6.3 (1.9–9.7)	0.346
Diabetes mellitus (%)	31.7	39.7	65.6*†	<0.001
Coronary artery disease (%)	3.2	11.1	28.1*†	<0.001
Cerebrovascular disease (%)	9.5	7.9	10.9	0.846
Body mass index (kg/m^2)	23.1 ± 3.1	23.8 ± 4.7	24.1 ± 3.6	0.344
Systolic blood pressure (mmHg)	150.3 ± 29.9	157.4 ± 28.7	159.3 ± 23.6	0.203
Heart rate (beat/min)	79.3 ± 11.3	79.8 ± 11.0	75.7 ± 11.1	0.075
ABI < 0.95 (%)	25.4	31.7	40.6	0.105
baPWV (cm/s)	1792.9 ± 533.9	2056.0 ± 546.4*	1921.7 ± 516.2	0.037
Cardiothoracic ratio (%)	48.1 ± 5.7	49.4 ± 5.9	51.5 ± 6.5*	0.007
AoAC	0 (0–3.8)	3 (0–7)	3 (0-7)*	0.003
Laboratory parameters				
Albumin (g/dL)	3.8 ± 0.3	3.8 ± 0.3	3.9 ± 0.3	0.846
Triglycerides (mg/dL)	125.0 (81–209)	111.0 (82–199)	142.5 (95.3–225.3)	0.647
Total cholesterol (mg/dL)	184.5 ± 39.2	172.7 ± 35.2	176.6 ± 45.1	0.247
Hemoglobin (g/dL)	10.4 ± 1.0	10.5 ± 1.3	10.6 ± 1.4	0.640
Creatinine (mg/dL)	9.9 ± 2.0	9.9 ± 2.5	8.9 ± 2.3*†	0.020
Calcium-phosphorous product (mg^2/dL^2)	40.1 ± 10.2	40.8 ± 12.7	44.5 ± 11.5	0.077
hs-CRP (mg/L)	1.9 (0.8–5.4)	2.9 (0.9–6.6)	3.0 (1.0–7.2)	0.554
Medications				
ACE inhibitors or ARBs (%)	11.1	15.9	39.1*†	<0.001
β-Blockers (%)	9.5	17.5	37.5*†	<0.001
Statins (%)	20.6	19.0	26.6	0.560
Echocardiographic data				
LAVI (mL/m^2)	27.9 ± 8.6	33.2 ± 10.6*	39.1 ± 12.7*†	<0.001
LVMI (g/m^2)	113.2 ± 31.1	136.1 ± 33.4*	157.6 ± 48.3*†	<0.001
LVEF (%)	68.2 ± 8.7	68.0 ± 8.0	63.8 ± 12.3*	0.019
Relative wall thickness	0.42 ± 0.09	0.43 ± 0.09	0.41 ± 0.12	0.607
E/E' ratio	9.3 ± 3.1	12.6 ± 5.1	21.2 ± 12.6*†	<0.001
E/A ratio	0.74 ± 0.20	0.84 ± 0.26	1.00 ± 0.44*†	<0.001
Deceleration time (ms)	179.9 ± 65.2	188.4 ± 51.7	194.9 ± 67.3	0.393
E' wave (cm/s)	7.3 ± 2.0	7.1 ± 2.5	5.6 ± 2.0*†	<0.001
E'sr (1/s)	1.5 ± 0.4	1.4 ± 0.3*	1.1 ± 0.3*†	<0.001
E/E'sr ratio (cm)	43.2 ± 5.4	57.8 ± 4.8*	92.1 ± 25.1*†	<0.001
GLS (%)	−18.4 ± 3.9	−17.2 ± 3.7	−14.9 ± 3.9*†	<0.001

ABI: ankle-brachial index; baPWV: brachial-ankle pulse wave velocity; AoAC: aortic arch calcification score; hs-CRP: high-sensitivity C-reactive protein; ACE: angiotensin-converting enzyme; ARB: angiotensin II receptor blocker; LAVI: left atrial volume index; LVMI: left ventricular mass index; LVEF: left ventricular ejection fraction; E: peak early transmitral filling wave velocity; E': early diastolic velocity of lateral mitral annulus; E'sr: global diastolic strain rate; GLS: global left ventricular longitudinal systolic strain. *$p < 0.05$ compared with the 1st tertile of the E/E'sr ratio; †$p < 0.05$ compared with the 2nd tertile of the E/E'sr ratio.

Table 4, the addition of the E/E' ratio to the basic model (comprises age, sex, dialysis vintage, DM, coronary artery disease, cerebrovascular disease, BMI, systolic blood pressure, ABI < 0.95, baPWV, cardiothoracic ratio, AoAC, album, triglycerides, total cholesterol, hemoglobin, creatinine, calcium-phosphorous product, hs-CRP, and the use of ACE inhibitors or ARBs, β-blockers, and statins) did not significantly improve the prediction for overall mortality, CV mortality,

Table 2: Comparison of baseline characteristics between patients with preserved GLS (≤−16%) or impaired GLS (>−16%).

Characteristics	Preserved GLS (≤−16%) ($n = 110$)	Impaired GLS (>−16%) ($n = 80$)	p
Age (year)	60.4 ± 11.8	61.4 ± 11.8	0.565
Male gender (%)	48.2	56.3	0.272
Dialysis vintage (year)	6.9 (2.6–12.5)	6.4 (2.5–10.2)	0.266
Diabetes mellitus (%)	34.5	61.3	<0.001
Coronary artery disease (%)	7.3	23.8	0.001
Cerebrovascular disease (%)	5.5	15.0	0.027
Body mass index (kg/m^2)	23.3 ± 3.4	24.2 ± 4.4	0.133
Systolic blood pressure (mmHg)	151.5 ± 27.4	160.8.4 ± 27.3	0.032
Heart rate (beat/min)	76.9 ± 11.2	80.1 ± 11.1	0.055
ABI < 0.95 (%)	24.5	43.8	0.039
baPWV (cm/s)	1876.3 ± 499.6	1983.9 ± 583.5	0.207
Cardiothoracic ratio (%)	49.6 ± 6.3	49.9 ± 6.1	0.744
AoAC	2 (0–5)	3 (0–7)	0.022
Laboratory parameters			
Albumin (g/dL)	3.8 ± 0.3	3.9 ± 0.3	0.584
Triglycerides (mg/dL)	115.5 (88.8–211)	136.5 (82–199.8)	0.942
Total cholesterol (mg/dL)	178.1 ± 41.1	177.7 ± 39.1	0.938
Hemoglobin (g/dL)	10.3 ± 1.1	10.7 ± 1.3	0.028
Creatinine (mg/dL)	9.7 ± 2.4	9.4 ± 2.2	0.404
Calcium-phosphorous product (mg^2/dL^2)	40.1 ± 11.6	44.2 ± 11.2	0.016
hs-CRP (mg/L)	2.2 (0.9–5.4)	3.1 (1.2–8.6)	0.227
Medications			
ACE inhibitors or ARBs (%)	19.1	26.3	0.240
β-Blockers (%)	20.0	23.8	0.535
Statins (%)	20.0	25.0	0.412
Echocardiographic data			
LAVI (mL/m^2)	34.6 ± 12.5	31.8 ± 10.3	0.113
LVMI (g/m^2)	129.9 ± 40.5	143.8 ± 43.7	0.026
LVEF (%)	69.9 ± 7.6	62.2 ± 11.2	<0.001
Relative wall thickness	0.42 ± 0.09	0.42 ± 0.11	0.788
E/E' ratio	13.3 ± 7.7	15.9 ± 11.4	0.077
E/A ratio	0.89 ± 0.29	0.82 ± 0.38	0.155
Deceleration time (ms)	190.0 ± 61.2	184.7 ± 62.9	0.788
E' wave (cm/s)	7.4 ± 2.2	5.7 ± 2.0	<0.001
E'sr (1/s)	1.55 ± 0.35	1.08 ± 0.24	<0.001
E/E'sr ratio (cm)	58.3 ± 21.3	73.0 ± 28.3	<0.001
GLS (%)	−19.6 ± 2.4	−13.1 ± 2.6	<0.001

ABI: ankle-brachial index; baPWV: brachial-ankle pulse wave velocity; AoAC: aortic arch calcification score; hs-CRP: high-sensitivity C-reactive protein; ACE: angiotensin-converting enzyme; ARB: angiotensin II receptor blocker; LAVI: left atrial volume index; LVMI: left ventricular mass index; LVEF: left ventricular ejection fraction; E: peak early transmitral filling wave velocity; E': early diastolic velocity of lateral mitral annulus; E'sr: global diastolic strain rate; GLS: global left ventricular longitudinal systolic strain.

and MACE. In contrast, the addition of the E/E'sr ratio to the basic model showed significant improvement of the prediction for overall mortality (χ^2 change = 13.914, $p < 0.001$), CV mortality (χ^2 change = 6.833, $p = 0.009$), and MACE (χ^2 change = 5.424, $p = 0.020$).

3.5. Comparison of LVEF and GLS to Overall Mortality, CV Mortality, and MACE. As shown by a direct comparison in Table 4, the addition of LVEF to the basic model did not significantly improve the outcome prediction. However, the addition of GLS to the basic model showed

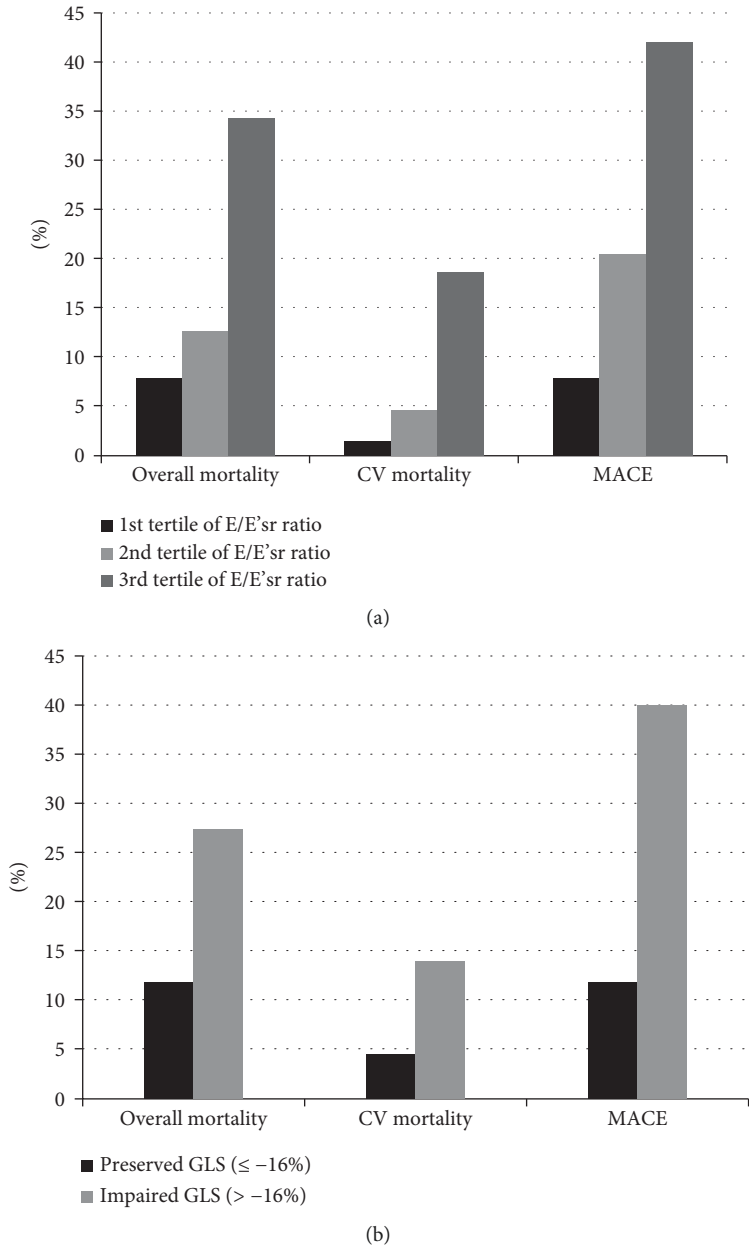

Figure 3: Incidence rates of overall mortality, CV mortality, and MACE over a median of 3.7 years among patients stratified by E/E'sr tertiles (a) and between patients with preserved GLS (≤−16%) or impaired GLS (>−16%) (b).

significant improvement of the prediction for overall mortality (χ^2 change = 12.007, $p = 0.001$), CV mortality (χ^2 change = 10.189, $p = 0.001$), and MACE (χ^2 change = 15.682, $p < 0.001$).

3.6. Incremental Value of the E/E'sr Ratio and GLS in Relation to Overall Mortality, CV Mortality, and MACE. The incremental values of the E/E'sr ratio and GLS in the prediction of overall mortality, CV mortality, and MACE are shown in Figures 6(a)–6(c), respectively. The addition of the echo model (comprises LAVI, LVMI, LVEF, and the E/E' ratio) to the basic model did not result in a significant improvement in the prediction of adverse outcomes. Moreover, the addition of the E/E'sr ratio and GLS to the basic model plus the echo model resulted in a further significant improvement in the prediction of overall mortality ($p < 0.001$), CV mortality ($p < 0.001$), and MACE ($p < 0.001$).

4. Discussion

In this study, we found that the higher E/E'sr ratio and GLS were independently associated with increased risk of overall mortality, CV mortality, and MACE in HD patients. The E/E'sr ratio was better than the E/E' ratio, and GLS was better than LVEF in predicting adverse outcomes. Furthermore, the E/E'sr ratio and GLS had significant incremental prognostic values beyond clinical and conventional echocardiographic parameters.

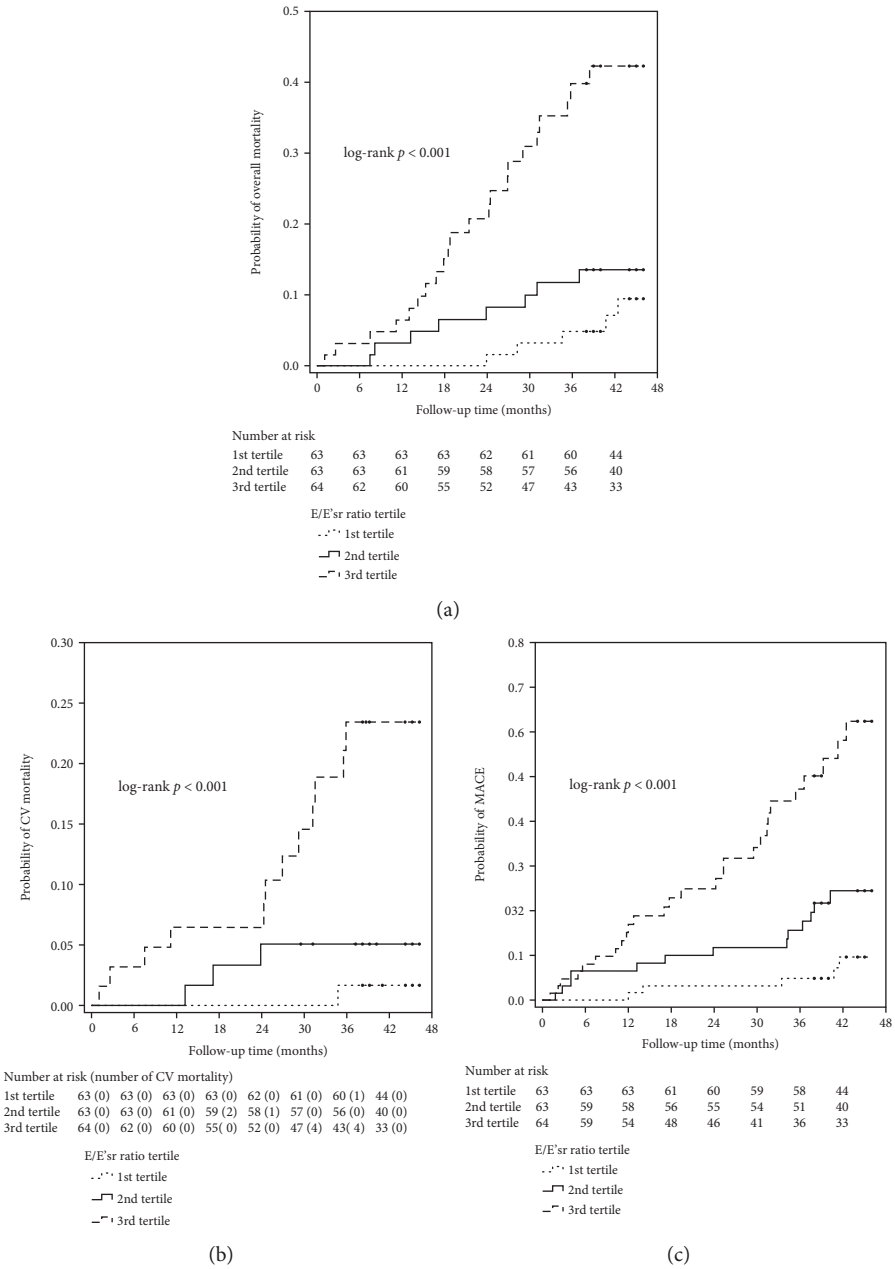

Figure 4: Kaplan-Meier curves for cumulative probability of overall mortality (log-rank $p < 0.001$) (a), CV mortality (log-rank $p < 0.001$) (b), and MACE (log-rank $p < 0.001$) (c) among patients stratified by E/E'sr tertiles.

An important finding of this study highlights that the E/E'sr ratio is a novel risk factor for overall mortality, CV mortality, and MACE in patients undergoing HD. At present, the E/E' ratio is a recommended modality to assess LV diastolic function [8, 28] and associated with mortality in HD patients [29] but it still has some drawbacks such as angle dependency and risk of errors with angulations > 20°. The E'sr obtained by STE from the whole left ventricle could overcome these limitations and more accurately represent global LV relaxation. Thus, the E/E'sr ratio correlates the LV filling pressures better than the E/E' ratio does [10, 11]. The deformation-based E/E'sr ratio provides more important information with regard to global myocardial relaxation than the velocity-based E/E' ratio, and the E/E'sr ratio was independently associated with adverse outcomes in several disease states and the general population [14, 30–32]. Furthermore, we found the superiority of the E/E'sr ratio over the E/E' ratio in predicting mortality and CV outcomes and the incremental prognostic value of the E/E'sr ratio and GLS over the conventional echocardiographic parameters in HD patients. The E'sr angle independently detects subtle myocardial motion, and it more precisely reflects LV global diastolic function compared with the E/E' ratio [32].

Evaluating LV systolic function is fundamental on echocardiography [6], and LVEF remains the most widely utilized indicator. Technical limitations in the measurement of LVEF

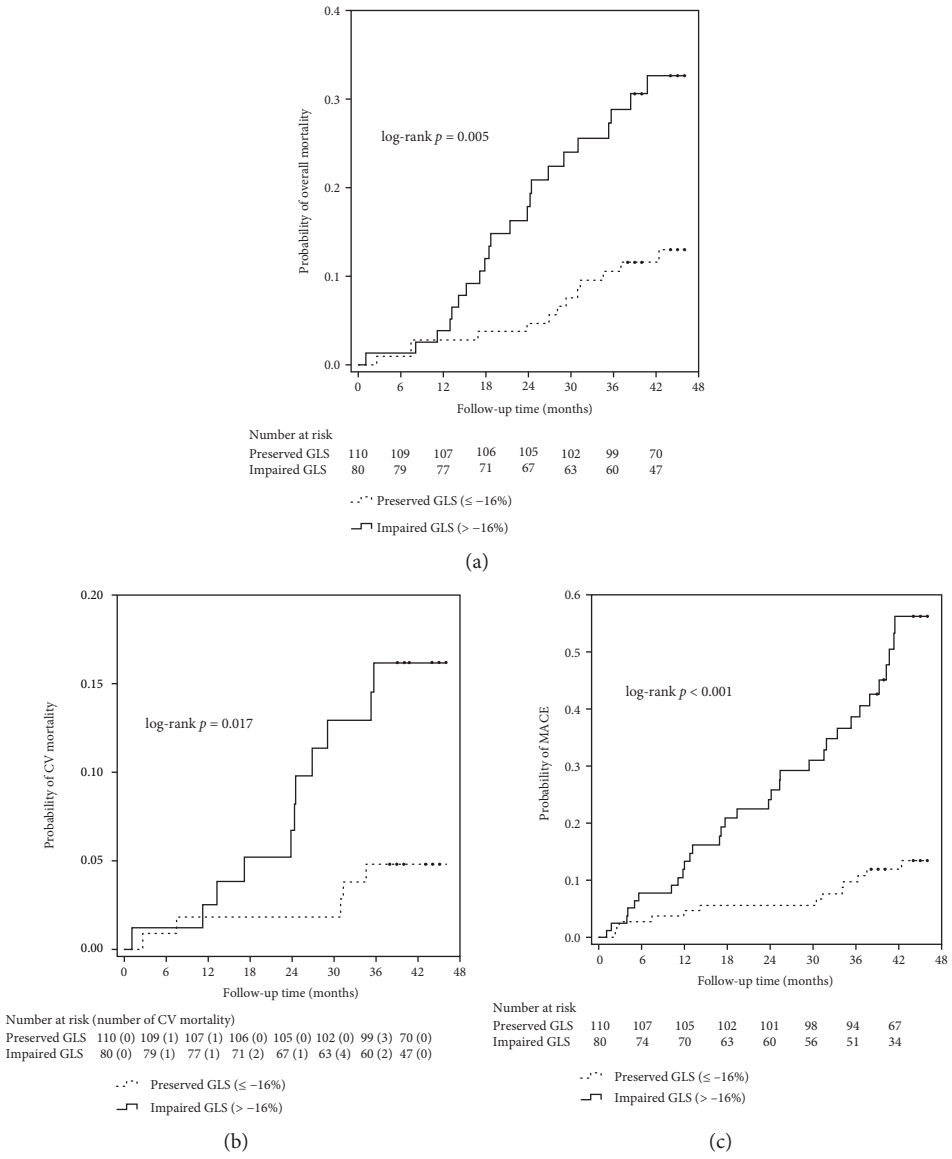

Figure 5: Kaplan-Meier curves for cumulative probability of overall mortality (log-rank $p = 0.005$) (a), CV mortality (log-rank $p = 0.017$) (b), and MACE (log-rank $p < 0.001$) (c) in patients with preserved GLS (≤−16%) or impaired GLS (>−16%).

include suboptimal endocardial definition and the formulas that make assumptions with regard to the geometry of the left ventricle [33]. Furthermore, LVEF as a measure of contractility is affected by load dependency and LVEF is insensitive to identify the subtle degree of systolic dysfunction in patients with LVEF > 45% [34]. As a result, the association between LVEF and mortality was inconsistent in certain studies [34, 35]. Our study indicates the independent effect of GLS on overall mortality, CV mortality, and MACE, and GLS outperformed LVEF in predicting unfavorable outcomes in chronic HD patients. These findings are in line with previous studies on nondialyzed CKD and on ESRD patients [16, 17, 27, 36]. GLS can assess the function of longitudinally orientated myofibers, which are most vulnerable because of their subendocardial location. Although GLS is load dependent, GLS is sensitive to detect early subendocardial changes with better reproducibility than LVEF and reflect the extent of myocardial fibrosis and uremic cardiomyopathy, even in those with preserved LVEF [16, 17, 37].

Another important finding is that the addition of the E/E'sr ratio and GLS to models containing markers of atherosclerosis and conventional indicators for LV systolic and diastolic functions as risk factors of mortality in HD patients [2, 21, 38, 39] offered incremental value in the prediction of adverse outcomes. The speckle tracking imaging is based on frame-by-frame tracking of the displacement of speckles within the myocardium during the cardiac cycle and subsequent measurement of LV deformations. This technique makes it independent on imaging factors including reverberation artifacts and attenuation. Thus, the E/E'sr ratio and GLS may be more representative of global LV function. Therefore, we suggest that the

Table 3: Associations of the E/E'sr ratio and GLS with overall mortality, CV mortality, and MACE using the Cox proportional hazards model.

	Overall mortality		CV mortality		MACE	
	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
E/E'sr ratio (per 10 cm)						
Crude	1.191 (1.090–1.301)	<0.001	1.271 (1.135–1.423)	<0.001	1.188 (1.101–1.282)	<0.001
Age and sex adjusted	1.185 (1.074–1.308)	0.001	1.261 (1.112–1.429)	<0.001	1.175 (1.083–1.274)	<0.001
Model 1 adjusted	1.392 (1.147–1.690)	0.001	1.476 (1.068–2.040)	0.018	1.188 (1.029–1.372)	0.019
Model 2 adjusted	1.484 (1.201–1.834)	<0.001	1.584 (1.058–2.371)	0.025	1.205 (1.040–1.397)	0.013
GLS (per 1%)						
Crude	1.127 (1.043–1.219)	0.003	1.202 (1.075–1.343)	0.001	1.174 (1.100–1.254)	<0.001
Age and sex adjusted	1.139 (1.046–1.239)	0.003	1.208 (1.072–1.360)	0.002	1.173 (1.095–1.255)	<0.001
Model 1 adjusted	1.266 (1.100–1.457)	0.001	1.312 (1.055–1.632)	0.015	1.212 (1.098–1.337)	<0.001
Model 2 adjusted	1.276 (1.101–1.480)	0.001	1.513 (1.088–2.104)	0.014	1.214 (1.103–1.337)	<0.001

CV: cardiovascular; MACE: major adverse cardiovascular events. Model 1 comprises age, sex, dialysis vintage, diabetes mellitus, coronary artery disease, cerebrovascular disease, body mass index, systolic blood pressure, ABI < 0.95, baPWV, cardiothoracic ratio, AoAC, albumin, triglycerides, total cholesterol, hemoglobin, creatinine, calcium-phosphorous product, and hs-CRP. Model 2 comprises model 1 plus the use of ACE inhibitors or ARBs, beta-blockers, and statins.

Table 4: Comparisons of the E/E' ratio with the E/E'sr ratio and LVEF with GLS in the prediction of overall mortality, CV mortality, and MACE.

	Overall mortality		CV mortality		MACE	
	χ^2 change	p	χ^2 change	p	χ^2 change	p
Basic model+E/E' ratio	0.509	0.476	0.005	0.943	0.422	0.516
Basic model+E/E'sr ratio	13.914	<0.001	6.833	0.009	5.424	0.020
Basic model+LVEF	0.484	0.487	1.234	0.267	1.167	0.280
Basic model+GLS	12.007	0.001	10.189	0.001	15.682	<0.001

p value was based on the incremental value compared with the basic model adjusted for age, sex, dialysis vintage, diabetes mellitus, coronary artery disease, cerebrovascular disease, body mass index, systolic blood pressure, ABI < 0.95, baPWV, cardiothoracic ratio, AoAC, albumin, triglycerides, total cholesterol, hemoglobin, creatinine, calcium-phosphorous product, hs-CRP, and the use of ACE inhibitors or ARBs, beta-blockers, and statins.

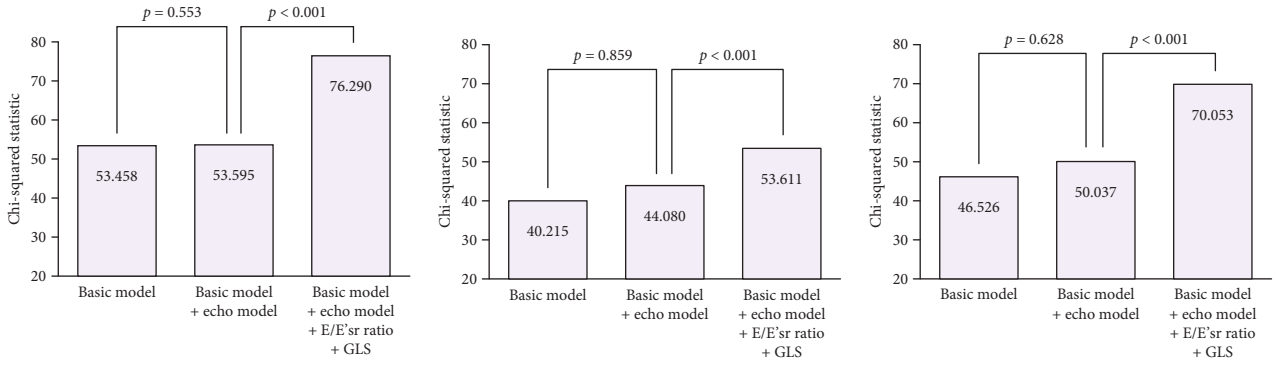

Figure 6: Addition of the E/E'sr ratio and GLS to the basic model and echo model improved the prediction of overall mortality (a), CV mortality (b), and MACE (c). Model χ^2 values are presented for a series of Cox models.

E/E'sr ratio and GLS should be measured during echocardiographic examinations to provide important prognostic information for chronic HD patients.

There are several limitations in the present study. First, the number of study patients is relatively small and the observation period may be not long enough. Second, the echocardiographic parameters were measured from the index beat. This method has been proved to be as accurate as the time-consuming method of averaging echocardiographic parameters from multiple cardiac cycles [9, 19]. Third, 2D STE generates longitudinal, radial, and circumferential deformation measurements and LV twist [40]. However, only E'sr and GLS were measured and analyzed in this study. The comparisons between these parameters with radial and circumferential strains and LV twist in predicting outcomes are warranted in the future study.

5. Conclusion

The E/E'sr ratio and GLS, as emerging indices of LV diastolic and systolic functions obtained from 2D STE, are useful parameters and are superior to the E/E' ratio and LVEF in the prediction of mortality and CV outcomes in maintenance HD patients and may offer an incremental value of prognostic significance over relevant clinical and conventional echocardiographic parameters.

Data Availability

The data supporting the findings of this study are available within the article or are available from the corresponding author upon reasonable request.

Disclosure

A part of this work has been presented as poster in the 2017 Annual General Meeting and Academic Lecture of the Taiwan Society of Internal Medicine.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

The research idea and study design were from J.C.H., H.M.S., and S.C.C.; data acquisition was performed by J.C.H., H.M.S., P.Y.W., J.J.L., W.H.L., S.C.C., and Y.W.C.; data analysis/interpretation was performed by J.C.H., H.M.S., J.J.L., and Y.W.C.; statistical analysis was performed by J.C.H., P.Y.W., and S.C.C.; supervision or mentorship was done by S.C.C., Y.L.H., J.M.C., and H.C.C. All authors contributed important intellectual content during manuscript drafting or revision and approved the final version of the manuscript.

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References

[1] R. Saran, B. Robinson, K. C. Abbott et al., "US Renal Data System 2018 Annual Data Report: epidemiology of kidney disease in the United States," *American Journal of Kidney Diseases*, vol. 73, no. 3, pp. A7–A8, 2019, Suppl 1, pp. Svii-Sxxii, S1-S772.

[2] S. C. Chen, J. C. Huang, H. M. Su et al., "Prognostic cardiovascular markers in chronic kidney disease," *Kidney and Blood Pressure Research*, vol. 43, no. 4, pp. 1388–1407, 2018.

[3] M. Cozzolino, M. Mangano, A. Stucchi, P. Ciceri, F. Conte, and A. Galassi, "Cardiovascular disease in dialysis patients," *Nephrology Dialysis Transplantation*, vol. 33, Supplement 3, pp. iii28–iii34, 2018, Suppl 3.

[4] C. Zoccali, U. Moissl, C. Chazot et al., "Chronic fluid overload and mortality in ESRD," *Journal of the American Society of Nephrology*, vol. 28, no. 8, pp. 2491–2497, 2017.

[5] S.-C. Hung, Y.-S. Lai, K.-L. Kuo, and D.-C. Tarng, "Volume overload and adverse outcomes in chronic kidney disease: clinical observational and animal studies," *Journal of the American Heart Association*, vol. 4, no. 5, article e001918, 2015.

[6] R. M. Lang, L. P. Badano, V. Mor-Avi et al., "Recommendations for cardiac chamber quantification by echocardiography in adults: an update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging," *European Heart Journal Cardiovascular Imaging*, vol. 16, no. 3, pp. 233–271, 2015.

[7] J. U. Voigt, G. Pedrizzetti, P. Lysyansky et al., "Definitions for a common standard for 2D speckle tracking echocardiography: consensus document of the EACVI/ASE/Industry Task Force to standardize deformation imaging," *European Heart Journal Cardiovascular Imaging*, vol. 16, no. 1, pp. 1–11, 2015.

[8] S. F. Nagueh, O. A. Smiseth, C. P. Appleton et al., "Recommendations for the evaluation of left ventricular diastolic function by echocardiography: an update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging," *European Heart Journal Cardiovascular Imaging*, vol. 17, no. 12, pp. 1321–1360, 2016.

[9] K. Kusunose, H. Yamada, S. Nishio et al., "Index-beat assessment of left ventricular systolic and diastolic function during atrial fibrillation using myocardial strain and strain rate," *Journal of the American Society of Echocardiography*, vol. 25, no. 9, pp. 953–959, 2012.

[10] H. Dokainish, R. Sengupta, M. Pillai, J. Bobek, and N. Lakkis, "Usefulness of new diastolic strain and strain rate indexes for the estimation of left ventricular filling pressure," *American Journal of Cardiology*, vol. 101, no. 10, pp. 1504–1509, 2008.

[11] K. Kimura, K. Takenaka, A. Ebihara et al., "Speckle tracking global strain rate E/E' predicts LV filling pressure more accurately than traditional tissue Doppler E/E'," *Echocardiography*, vol. 29, no. 4, pp. 404–410, 2012.

[12] J. Wang, D. S. Khoury, V. Thohan, G. Torre-Amione, and S. F. Nagueh, "Global diastolic strain rate for the assessment of left ventricular relaxation and filling pressures," *Circulation*, vol. 115, no. 11, pp. 1376–1383, 2007.

[13] M. Ersboll, M. J. Andersen, N. Valeur et al., "Early diastolic strain rate in relation to systolic and diastolic function and prognosis in acute myocardial infarction: a two-dimensional speckle-tracking study," *European Heart Journal*, vol. 35, no. 10, pp. 648–656, 2014.

[14] Y. H. Chan, H. F. Lee, L. S. Wu et al., "Ratio of transmitral early filling velocity to early diastolic strain rate predicts outcomes in patients with systolic heart failure," *European Heart Journal Cardiovascular Imaging*, vol. 18, no. 1, pp. 79–85, 2017.

[15] M. S. Amzulescu, M. De Craene, H. Langet et al., "Myocardial strain imaging: review of general principles, validation, and sources of discrepancies," *European Heart Journal Cardiovascular Imaging*, vol. 20, no. 6, pp. 605–619, 2019.

[16] Y. W. Liu, C. T. Su, J. M. Sung et al., "Association of left ventricular longitudinal strain with mortality among stable hemodialysis patients with preserved left ventricular ejection fraction," *Clinical Journal of the American Society of Nephrology*, vol. 8, no. 9, pp. 1564–1574, 2013.

[17] R. Kramann, J. Erpenbeck, R. K. Schneider et al., "Speckle tracking echocardiography detects uremic cardiomyopathy early and predicts cardiovascular mortality in ESRD," *Journal*

of the American Society of Nephrology, vol. 25, no. 10, pp. 2351–2365, 2014.

[18] K. Negishi, T. Negishi, K. Kurosawa et al., "Practical guidance in echocardiographic assessment of global longitudinal strain," *Journal of the American College of Cardiology Cardiovascular Imaging.*, vol. 8, no. 4, pp. 489–492, 2015.

[19] M. Govindan, A. Kiotsekoglou, S. K. Saha et al., "Validation of echocardiographic left atrial parameters in atrial fibrillation using the index beat of preceding cardiac cycles of equal duration," *Journal of the American Society of Echocardiography*, vol. 24, no. 10, pp. 1141–1147, 2011.

[20] C. S. Lee, T. H. Lin, P. C. Hsu et al., "Measuring left ventricular peak longitudinal systolic strain from a single beat in atrial fibrillation: validation of the index beat method," *Journal of the American Society of Echocardiography*, vol. 25, no. 9, pp. 945–952, 2012.

[21] S.-C. Chen, M.-Y. Lee, J.-C. Huang, M.-C. Shih, J.-M. Chang, and H.-C. Chen, "Association of ankle-brachial index and aortic arch calcification with overall and cardiovascular mortality in hemodialysis," *Scientific Reports*, vol. 6, no. 1, article 33164, 2016.

[22] F. G. Fowkes, J. F. Price, M. C. Stewart et al., "Aspirin for prevention of cardiovascular events in a general population screened for a low ankle brachial index: a randomized controlled trial," *Journal of the American Medical Association*, vol. 303, no. 9, pp. 841–848, 2010.

[23] X. Guo, J. Li, W. Pang et al., "Sensitivity and specificity of ankle-brachial index for detecting angiographic stenosis of peripheral arteries," *Circulation Journal*, vol. 72, no. 4, pp. 605–610, 2008.

[24] T. Ogawa, H. Ishida, N. Matsuda et al., "Simple evaluation of aortic arch calcification by chest radiography in hemodialysis patients," *Hemodialysis International*, vol. 13, no. 3, pp. 301–306, 2009.

[25] J. C. Huang, I. C. Kuo, Y. C. Tsai et al., "Heart rate variability predicts major adverse cardiovascular events and hospitalization in maintenance hemodialysis patients," *Kidney and Blood Pressure Research*, vol. 42, no. 1, pp. 76–88, 2017.

[26] T. H. Marwick, R. L. Leano, J. Brown et al., "Myocardial strain measurement with 2-dimensional speckle-tracking echocardiography: definition of normal range," *Journal of the American College of Cardiology: Cardiovascular Imaging*, vol. 2, no. 1, pp. 80–84, 2009.

[27] R. Krishnasamy, N. M. Isbel, C. M. Hawley et al., "Left ventricular global longitudinal strain (GLS) is a superior predictor of all-cause and cardiovascular mortality when compared to ejection fraction in advanced chronic kidney disease," *PLoS One*, vol. 10, no. 5, article e0127044, 2015.

[28] Y. W. Liu, C. T. Su, E. J. Song et al., "The role of echocardiographic study in patients with chronic kidney disease," *Journal of the Formosan Medical Association*, vol. 114, no. 9, pp. 797–805, 2015.

[29] J. C. Huang, Y. C. Tsai, P. Y. Wu et al., "Independent association of overhydration with all-cause and cardiovascular mortality adjusted for global left ventricular longitudinal systolic strain and E/E' ratio in maintenance hemodialysis patients," *Kidney and Blood Pressure Research*, vol. 43, no. 4, pp. 1322–1332, 2018.

[30] P. C. Hsu, W. H. Lee, C. Y. Chu et al., "The ratio of early mitral inflow velocity to global diastolic strain rate as a useful predictor of cardiac outcomes in patients with atrial fibrillation," *Journal of the American Society of Echocardiography*, vol. 27, no. 7, pp. 717–725, 2014.

[31] J. S. Dahl, S. Barros-Gomes, L. Videbaek et al., "Early diastolic strain rate in relation to systolic and diastolic function and prognosis in aortic stenosis," *Journal of the American College of Cardiology: Cardiovascular Imaging*, vol. 9, no. 5, pp. 519–528, 2016.

[32] M. C. H. Lassen, S. R. Biering-Sørensen, F. J. Olsen et al., "Ratio of transmitral early filling velocity to early diastolic strain rate predicts long-term risk of cardiovascular morbidity and mortality in the general population," *European Heart Journal*, vol. 40, no. 6, pp. 518–525, 2019.

[33] C. Szymanski, F. Lévy, and C. Tribouilloy, "Should LVEF be replaced by global longitudinal strain?," *Heart*, vol. 100, no. 21, pp. 1655-1656, 2014.

[34] J. P. Curtis, S. I. Sokol, Y. Wang et al., "The association of left ventricular ejection fraction, mortality, and cause of death in stable outpatients with heart failure," *Journal of the American College of Cardiology*, vol. 42, no. 4, pp. 736–742, 2003.

[35] J. S. Gottdiener, R. L. McClelland, R. Marshall et al., "Outcome of congestive heart failure in elderly persons: influence of left ventricular systolic function: The Cardiovascular Health Study," *Annals of Internal Medicine*, vol. 137, no. 8, pp. 631–639, 2002.

[36] R. Krishnasamy, N. M. Isbel, C. M. Hawley et al., "The association between left ventricular global longitudinal strain, renal impairment and all-cause mortality," *Nephrology Dialysis Transplantation*, vol. 29, no. 6, pp. 1218–1225, 2014.

[37] V. F. Panoulas, S. Sulemane, K. Konstantinou et al., "Early detection of subclinical left ventricular myocardial dysfunction in patients with chronic kidney disease," *European Heart Journal Cardiovascular Imaging*, vol. 16, no. 5, pp. 539–548, 2015.

[38] S.-C. Chen, J.-M. Chang, S.-J. Hwang et al., "Ankle brachial index as a predictor for mortality in patients with chronic kidney disease and undergoing haemodialysis," *Nephrology*, vol. 15, no. 3, pp. 294–299, 2010.

[39] S. Y. Wei, J. C. Huang, S. C. Chen, J. M. Chang, and H. C. Chen, "Unequal arterial stiffness with overall and cardiovascular mortality in patients receiving hemodialysis," *The American Journal of the Medical Sciences*, vol. 351, no. 2, pp. 187–193, 2016.

[40] S. A. Luis, J. Chan, and P. A. Pellikka, "Echocardiographic assessment of left ventricular systolic function: an overview of contemporary techniques, including speckle-tracking echocardiography," *Mayo Clinic Proceedings*, vol. 94, no. 1, pp. 125–138, 2019.

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Research Article

A Low Ankle-Brachial Index and High Brachial-Ankle Pulse Wave Velocity Are Associated with Poor Cognitive Function in Patients Undergoing Hemodialysis

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Patients with end-stage renal disease (ESRD) have an increased risk of both impaired cognitive function and peripheral artery disease (PAD) than the general population. The association between PAD and dementia is recognized, but there are limited studies in patients with ESRD. The aim of this study was to evaluate the relationship between ankle-brachial index (ABI) and brachial-ankle pulse wave velocity (baPWV) and cognitive impairment in patients receiving hemodialysis (HD). We enrolled 136 prevalent HD patients (mean age 59.3 ± 10.5 years, 55.9% male). Cognitive performance was measured using the Montreal Cognitive Assessment (MoCA) and Cognitive Abilities Screening Instrument (CASI) by trained psychiatrists. Associations between the cognitive function and ABI and baPWV were assessed using multiple linear regression analysis. Compared with HD patients with ABI ≥ 0.9, patients with ABI < 0.9 had lower MoCA score ($p = 0.027$) and lower CASI score but did not achieve significant level ($p = 0.056$). In the multivariate stepwise linear regression analysis, ABI (per 0.1) was independently positively associated with the MoCA score (β coefficient = 0.62, $p = 0.011$) and the CASI score (β coefficient = 1.43, $p = 0.026$). There is a negative association between baPWV (per 100 cm/s) and CASI (β coefficient = −0.70, $p = 0.009$). In conclusion, a low ABI or high baPWV was associated with a lower cognitive function in HD patients.

1. Introduction

Patients with chronic kidney disease (CKD) or end-stage renal disease (ESRD) have a higher risk of dementia than the general population [1, 2], and patients with both dementia and ESRD have been associated with disability, hospitalization, dialysis withdrawal, and mortality [3–5]. As such, cognition should be evaluated in patients with ESRD to detect vascular and nonvascular dementia and prevent cognitive decline and its consequences. Given the high prevalence of dementia in patients with ESRD, identifying clinical markers that can predict cognitive dysfunction may be beneficial for both prevention and reducing health care costs. Scuteri et al. reported an association between arterial stiffness and brain injury and related brain pathologies [6]. They concluded that increased central pulse pressure and

wave reflections may influence both the brain and kidneys and that it is likely that these phenomena are emphasized in ESRD patients.

Patients with ESRD have a high prevalence of peripheral artery disease (PAD), an important manifestation of systemic atherosclerosis [7]. PAD shares similar risk factors with coronary artery disease and cerebrovascular disease [8]. The ankle-brachial index (ABI) and pulse wave velocity (PWV) are common noninvasive tools used to quantitatively assess arterial health with regard to blocked arteries and arterial stiffness, respectively. PAD has been associated with an increased risk of both cardiovascular disease [9] and cognitive dysfunction in the general population [10], and a low ABI has been reported to predict the future risk of cognitive impairment [11] and dementia [12].

However, few studies have evaluated the association between PAD and cognitive function in patients with CKD or ESRD [13, 14], despite being at high risk of atherosclerosis and dementia. Moreover, no previous study has simultaneously evaluated ABI and PWV as markers of cognitive function in ESRD patients. A better understanding of the relationship between PAD and cognitive decline in ESRD patients may provide new insights into the physiological mechanisms of the prodromal stage of dementia. Accordingly, the aim of this study was to assess the association between ABI and brachial-ankle PWV (baPWV) and cognitive function in patients receiving hemodialysis (HD).

2. Methods

2.1. Study Subjects and Design. This study was conducted at a dialysis clinic in a regional hospital in southern Taiwan from August 2016 to January 2017. All patients received regular HD three times per week with high-flux dialyzers and a blood flow rate of 250-300 mL/min, dialysate flow rate of 500 mL/min, with each HD session lasting for 3.5-4 hours. Patients aged > 30 years who had received maintenance dialysis for at least 90 days were recruited. After excluding the patients who refused to undergo ABI-form device ($n = 5$) or neuropsychological ($n = 17$) examinations, patients with atrial fibrillation ($n = 4$), patients with bilateral below-the-knee amputations ($n = 3$), and patients who had been hospitalized for 4 weeks prior to study enrollment ($n = 5$), the remaining 136 patients (76 men and 60 women) were included into the final analysis. The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUHIRB-E(I)-20160095), and written informed consent was obtained from all patients. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Demographic, Medical, and Laboratory Data. Demographic and medical data including sex, age, smoking history (ever versus never), and comorbidities were obtained from interviews and the patients' medical records. Body mass index was calculated as weight divided by height squared in kg/m^2. Hypertension was defined as blood pressure ≥ 140/90 mmHg or taking antihypertensive drugs, and diabetes was defined as fasting blood glucose level of ≥126 mg/dL or taking antidiabetic drugs. Patients with a history of cerebrovascular accidents, including cerebral bleeding and infarction, were defined as having a cerebrovascular disease, and those with a history of angina, ischemic changes in electrocardiography, old myocardial infarction, or coronary bypass surgery/angioplasty were defined as having coronary artery disease. Laboratory examinations were performed in fasting blood samples obtained ≤1 month of enrollment using an autoanalyzer (COBAS Integra 400, Roche Diagnostics GmbH, Mannheim, Germany).

2.3. Measurement of ABI and baPWV. Because ABI and baPWV might be influenced by hemodialysis [15], all the values of ABI and baPWV were measured 10–30 minutes before hemodialysis. The values of ABI and baPWV were measured by using an ABI-form device (VP1000; Colin Co. Ltd., Komaki, Japan), which automatically and simultaneously measures blood pressures in both arms and ankles using an oscillometric method [16]. Occlusion and monitoring cuffs were placed tightly around the upper arms without blood access and both sides of the lower extremities in the supine position. ABI was calculated by the ratio of the ankle systolic blood pressure divided by the arm systolic blood pressure, and the lower value of the ankle systolic blood pressure was used for the calculation. For measuring baPWV, pulse waves obtained from the brachial and tibial arteries were recorded simultaneously, and the transmission time, which was defined as the time interval between the initial increase in brachial and tibial waveforms, was determined. The transmission distance from the arm to each ankle was calculated according to body height. The baPWV value was automatically computed as the transmission distance divided by the transmission time. After obtaining bilateral baPWV values, the highest one was used as a representative for each subject. The ABI and baPWV measurements were done once in each patient. Concerning the reproducibility of ABI and baPWV, we randomly evaluated 25 patients at least 15 minutes apart for the reproducibility of ABI and baPWV by using 2 separate measurements. Mean percent error was calculated as the absolute difference divided by the average of the 2 observations.

2.4. Cognitive Function Assessment. Three cognitive function tests evaluated in this study were Montreal Cognitive Assessment (MoCA) [17, 18] and Cognitive Abilities Screening Instrument (CASI) [18, 19] (Supplementary Table 1). The MoCA is a more sensitive 30-point screening tool that includes copying a cube, verbal abstraction, serial subtraction, drawing a clock, a 5-word learning and delayed recall task, naming an animal, digit span backward and forwards, selective attention, repeating a sentence, phonemic word fluency, and spatial and temporal orientation. These tasks encompass multiple domains of cognition, including short-term memory, visuospatial ability, and executive function, language, attention, concentration and working memory, and orientation to time and place. The CASI assesses a broad range of cognitive domains using a 40-item global cognitive test, which contains nine cognitive

evaluation domains, including long-term memory, short-term memory, orientation, attention, mental manipulation, list-generating fluency, language, abstraction/judgment, and drawing (Supplementary Table 1).

2.5. Statistical Analysis. Descriptive statistics were presented as percentages, means ± standard deviations, or medians (25^{th}-75^{th} percentile) for HD duration and triglycerides. Differences between groups were analyzed using the chi-square test for categorical variables and the independent t-test for continuous variables with approximately normal distribution or the Mann-Whitney U test for continuous variables with skewed distribution. Multiple stepwise linear regression analyses were used to identify the factors associated with cognitive function (MoCA and CASI). The adjusted multivariate variables in the models included age, sex, smoking, a history of hypertension, diabetes, cerebrovascular and coronary artery diseases, systolic and diastolic blood pressures, body mass index, log-transformed hemodialysis duration, cause of end-stage renal disease, albumin, log-transformed triglyceride, total cholesterol, hemoglobin, creatinine, calcium-phosphorus product, Kt/V, and amount of ultrafiltration. Relevant demographic parameters were also analyzed by a backward stepwise selection with p values for independent variables to enter and to stay in the models set at 0.1 and subsequently a final elimination step at $p < 0.05$. All statistical analyses were performed using SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA) and STATA version 14 (StataCorp LP, College Station, TX, USA). A two-tailed p value of <0.05 was considered to be statistically significant.

3. Results

3.1. Demographic and Clinical Characteristics. The mean age of the 136 HD patients was 59.3 ± 10.5 years, 55.9% were men, and 21.3% had an ABI < 0.9. The mean percent error for ABI and baPWV measurement was 3.58 ± 3.15% and 5.8% ± 5.0%, respectively. The patients were stratified into two groups according to ABI < 0.9 or ≥0.9. Comparisons of the clinical characteristics between the two groups are shown in Table 1. Compared to the ABI ≥ 0.9 groups, the ABI < 0.9 groups were older and had more diabetes-related comorbidities, lower systolic and diastolic blood pressures, and higher levels of calcium-phosphorus products. Regarding cognitive function, compared to the ABI ≥ 0.9 groups, the ABI < 0.9 groups had lower MoCA ($p = 0.027$) scores. The ABI < 0.9 group also had a lower CASI score, but the difference was not significant ($p = 0.056$).

3.2. Associations of ABI and Cognitive Function in HD Patients. In univariate linear regression models, ABI was positively associated with MoCA (β coefficient 1.07, 95% confidence interval (CI) 0.55 to 1.59, and $p < 0.001$) and CASI (β coefficient 2.87, 95% CI 1.45 to 4.29, and $p < 0.001$) (Table 2). In multivariate linear regression models with stepwise backward covariate selection, ABI was persisted positively associated with MoCA (β coefficient 0.62, 95% CI 0.14 to 1.09, and $p = 0.011$) and CASI (β coefficient 1.43, 95% CI 0.17 to 2.70, and $p = 0.026$) (Table 2). The stepwise backward covariate selection models of ABI and cognitive function (MoCA and CASI) were demonstrated in Supplementary Tables 2-4. Since extremely high ABI (>1.3) is correlated to multiple comorbidities or arterial calcification, we reanalyzed the association between ABI and cognitive function test after excluding subjects with ABI value > 1.3. Similar results found the positive association between ABI and the MoCA or CASI score in univariate linear regression analysis (β coefficient 1.08, 95% CI 0.55 to 1.61, and $p < 0.001$ in MoCA; β coefficient 2.97, 95% CI 1.53 to 4.42, and $p < 0.001$ in CASI) and multivariate stepwise linear regression analysis (β coefficient 0.63, 95% CI 0.15 to 1.11, and $p = 0.011$ in MoCA; β coefficient 1.36, 95% CI 0.04 to 2.69, and $p = 0.043$ in CASI).

3.3. Associations of baPWV and Cognitive Function in HD Patients. In univariate linear regression models, baPWV was negatively associated with MoCA (β coefficient -0.33, 95% CI -0.56 to -0.11, and $p = 0.004$) and CASI (β coefficient -1.16, 95% CI -1.76 to -0.56, and $p < 0.001$) (Table 3). In multivariate linear regression models with stepwise backward covariate selection, baPWV was persisted negatively associated with CASI (β coefficient -0.70, 95% CI -1.22 to -0.18, and $p = 0.009$) but not with MoCA (β coefficient -0.075, 95% CI -0.31 to 0.16, and $p = 0.52$) (Table 3). The stepwise backward covariate selection models of baPWV and cognitive function (MoCA and CASI) were demonstrated in Supplementary Tables 5-7. Since both sides of PAD could be found in patients with HD that influence the baPWV data, we reanalyzed the association between baPWV and cognitive function test after excluding subjects with ABI value < 0.9. Similar results found the negative association between baPWV and MoCA (β coefficient -0.38, 95% CI -0.63 to -0.12, and $p = 0.004$) or CASI score (β coefficient -1.01, 95% CI -1.65 to -0.37, and $p = 0.002$) in the univariate linear regression analysis. However, the insignificant negative association was demonstrated in stepwise linear regression approach (β coefficient -0.20, 95% CI -0.45 to 0.06, and $p = 0.13$ in MoCA; β coefficient -0.44, 95% CI -1.07 to 0.18, and $p = 0.16$ in CASI).

3.4. Subgroup Analysis of ABI or baPWV and Cognitive Function in HD Patients. Subgroup analysis of gender and baseline comorbidities demonstrated that ABI was positively associated with cognitive function except for the male gender, smoking habit, and patients with hypertension and stroke comorbidities (Figure 1). The baPWV was negatively associated with cognitive function except for male, smoking habit, patients with/without diabetes comorbidity, no hypertension comorbidity, or those with a stroke history in MoCA. A similar pattern was found in the CASI test (Figure 2).

3.5. Sensitivity Analysis of ABI or baPWV and Cognitive Function in HD Patients. We evaluated the comorbidities and cognitive function test (MoCA and CASI) association. Only diabetes was negatively associated with cognitive function test score (Supplementary Table 6). Considering diabetes as an important confounder for cognitive function, sensitivity analysis of stepwise regression models with

Table 1: Comparison of baseline characteristics in patients categorized by ABI < 0.9 or ≥0.9.

Characteristics	All patients ($n = 136$)	ABI ≥ 0.9 ($n = 107$)	ABI < 0.9 ($n = 29$)	p
Age	59.3 ± 10.5	58.1 ± 9.8	63.7 ± 12.1	0.011
Male gender (%)	55.9	57.9	48.3	0.352
Smoking history (%)	37.3	37.4	37.0	0.973
Diabetes mellitus (%)	42.5	36.4	66.7	0.005
Hypertension (%)	56.7	55.1	63.0	0.463
Coronary artery disease (%)	7.5	6.5	11.1	0.419
Cerebrovascular disease (%)	6.0	4.7	11.1	0.207
Systolic blood pressure (mmHg)	156.2 ± 24.5	158.8 ± 23.0	146.5 ± 27.9	0.016
Diastolic blood pressure (mmHg)	82.0 ± 14.5	84.8 ± 13.5	71.5 ± 13.3	<0.001
Body mass index (kg/m^2)	24.0 ± 3.9	23.9 ± 4.0	24.2 ± 3.2	0.734
ABI	0.98 ± 0.19	1.06 ± 0.10	0.69 ± 0.14	<0.001
baPWV (cm/s)	1870.4 ± 441.4	1897.5 ± 389.1	1768.7 ± 593.7	0.164
Duration of hemodialysis (years)	7.5 (3.1-13.0)	7.8 (3.1-13.0)	5.9 (3.1-12.5)	0.650
Cause of end-stage renal disease				0.289
Hypertension (%)	5.9	5.6	6.9	
Diabetes mellitus (%)	40.4	36.4	55.2	
Glomerulonephritis (%)	49.3	53.3	34.5	
Others* (%)	4.4	4.7	3.4	
Laboratory parameters				
Albumin (g/dL)	3.9 ± 0.3	3.9 ± 0.3	3.8 ± 0.3	0.216
Triglyceride (mg/dL)	124.5 (83-195.5)	120.5 (82.75-189.5)	138.5 (83.25-234.25)	0.277
Total cholesterol (mg/dL)	173.6 ± 40.6	172.8 ± 39.6	176.8 ± 44.7	0.638
Hemoglobin (g/dL)	10.5 ± 1.4	10.5 ± 1.4	10.8 ± 1.4	0.252
Creatinine (mg/dL)	10.1 ± 2.1	10.1 ± 2.1	9.9 ± 2.2	0.606
CaXP product (mg^2/dL^2)	45.5 ± 11.8	44.4 ± 11.1	49.6 ± 13.8	0.038
Dialysis dose (Kt/V)	1.62 ± 0.26	1.62 ± 0.26	1.60 ± 0.28	0.775
Total net fluid loss (amount of ultrafiltration) (kg)	2.70 ± 1.08	2.74 ± 1.04	2.53 ± 1.26	0.356
Cognitive function assessment				
MoCA (score)	18.7 ± 6.0	19.5 ± 5.3	16.0 ± 7.5	0.027
CASI (score)	75.6 ± 16.4	77.5 ± 13.5	68.5 ± 23.3	0.056

Abbreviation: ABI: ankle-brachial index; baPWV: brachial-ankle pulse wave velocity; CaXP product: calcium × phosphorus product; MoCA: Montreal Cognitive Assessment; CASI: Cognitive Abilities Screening Instrument. *Other causes of end-stage renal disease include polycystic kidney disease, tumor, systemic lupus erythematosus, gout, and interstitial nephritis.

additional diabetes comorbidity adjustment in ABI or baPWV and cognitive function was analyzed. A similar result was found (Supplementary Tables 7 and 8).

4. Discussion

This study examined the relationship between PAD and cognitive performance in 136 prevalent HD patients. PAD was assessed according to ABI and baPWV, and cognitive performance was assessed according to MoCA and CASI, which collectively evaluated memory, orientation, attention, visual screening, motor speed, planning abilities, executive function, and language. A low ABI was associated with low MoCA and CASI scores, and a high baPWV was associated with low CASI scores. Since cognition was evaluated using questionnaires with different sensitivities and specificities, it would be promising results to find the association between ABI or baPWV and cognition in HD patients.

The prevalence of cognitive impairment is high in patients undergoing HD [20–22]. Considering the different definition of cognitive impairment in patients with kidney disease based on sensitivity and specificity, MoCA is a valid, more sensitive, and well-suited screening tool for cognitive impairment in patients receiving HD [23, 24]. The optimal cut-off of ≤24 points out of a 30-point maximum is lower than the cut-off value of ≤26 described in the original data collected in a population of patients with Alzheimer's disease and mild cognitive impairment [25]. In the present HD cohort, there is a higher prevalence of cognitive impairment (84.6%; 114/136 based on MoCA cut-off ≤ 24 points) than

Table 2: The association between ABI and cognitive function test (MoCA and CASI) using univariate linear regression analysis and multivariate stepwise linear regression analysis.

Cognitive function test	Univariate		Multivariate (stepwise)*	
	β coefficient (95% CI)	p value	β coefficient (95% CI)	p value
MoCA	1.07 (0.55 to 1.59)	<0.001	0.62 (0.14 to 1.09)	0.011
CASI	2.87 (1.45 to 4.29)	<0.001	1.43 (0.17 to 2.70)	0.026

*Adjusting for stepwise procedure selected covariates (age, sex, smoking habit, a history of diabetes, hypertension, coronary artery disease, and cerebrovascular disease, systolic and diastolic blood pressures, body mass index, log-transformed hemodialysis duration, cause of end-stage renal disease, albumin, log-transformed triglyceride, total cholesterol, hemoglobin, creatinine and calcium-phosphorus product, Kt/V, and amount of ultrafiltration).

Table 3: The association between baPWV and cognitive function test (MoCA and CASI) using univariate linear regression analysis and multivariate stepwise linear regression analysis.

Cognitive function test	Univariate		Multivariate (stepwise)*	
	β coefficient (95% CI)	p value	β coefficient (95% CI)	p value
MoCA	-0.33 (-0.56 to -0.11)	0.004	-0.075 (-0.31 to 0.16)	0.52
CASI	-1.16 (-1.76 to -0.56)	<0.001	-0.70 (-1.22 to -0.18)	0.009

*Adjusting for stepwise procedure selected covariates (age, sex, smoking habit, a history of diabetes, hypertension, coronary artery disease, and cerebrovascular disease, systolic and diastolic blood pressures, body mass index, log-transformed hemodialysis duration, cause of end-stage renal disease, albumin, log-transformed triglyceride, total cholesterol, hemoglobin, creatinine and calcium-phosphorus product, Kt/V, and amount of ultrafiltration).

other HD cohorts [26]. This may be related to longer dialysis vintage and higher diabetes mellitus comorbidity in the HD cohort.

The first important finding of this study is that a low ABI was associated with poor cognitive function. The etiology for cognitive impairment in HD patients is complex, including traditional risk factors (old age, hypertension, diabetes, and smoking), nontraditional risk factors (anemia, albuminuria, inflammation, homocysteinemia, and malnutrition), and dialysis-related factors (hypotension, all of which can lead to chronic cerebral hypoxemia or edema) [27, 28]. In addition, high rates of small and large cerebral vascular injuries, including white matter lesions, subcortical atrophy, lacunar infarcts, and microbleeds, have been reported in patients with CKD in brain imaging studies [29, 30]. Vascular stiffness and abnormalities in structure and function have also been associated with cognitive decline and stroke [31]. The ABI, an easily obtained good marker of atherosclerosis [32, 33], has been shown to be a good prognostic marker of atherosclerosis for stroke. Subjects with a low ABI have been reported to have more atherosclerotic and vascular conditions, and these conditions may lead to generalized endothelial dysfunction [34], stenosis of intracranial and extracranial arteries [35], and decreased cerebral perfusion inducing oxidative stress [36]. Taken together, these findings may explain the association between low ABI and poor overall cognitive function [37]. A low ABI has also been correlated with global cortical thinning and reduced cortical thickness in the limbic, parietal, temporal, and occipital lobes [38]. A decrease in overall cerebral perfusion due to atherosclerosis may cause pathological changes such as infarcts or neurodegeneration [39]. The present study declared that a low ABI was associated with the risk of poor cognitive function in HD patients, as indicated by low MoCA and CASI scores.

The second important finding of this study is that a high baPWV was associated with poor cognitive function. Arterial stiffness is a marker of functional and structural changes in the arteries and can contribute to microvascular brain diseases, and it can be assessed noninvasively using PWV measurements. Several studies have examined the association between arterial stiffness and cognitive function in the general population [40, 41] and in patients with CKD or ESRD [13, 14]. Longitudinal studies have indicated that high values of PWV can predict lower cognitive function score in the elderly [42]. One study with 72 ESRD patients undergoing HD showed that a higher carotid-femoral PWV was associated with cognitive impairment [14]. Another study suggests an inverse association between baPWV and cognitive function among the elderly [43]. The present study demonstrated a negative association between baPWV and cognitive function score (CASI). Thus, baPWV could be considered to be a marker of cognition in ESRD patients.

In the present study, diabetes is significantly negatively associated with the cognitive function test score. However, previous cerebrovascular event history is not significantly negatively associated with the cognitive function test score because of a few patients with old stroke hospitalization records in our study. Thus, sample sizes are insufficient to found the association. Therefore, the main results were analyzed by multivariate stepwise variable selection model in this study. Stepwise regression is a method of fitting regression models in which the choice of predictive variables is carried out by an automatic procedure. At each step, a variable is considered for addition to or subtraction from the set of explanatory variables based on some prespecified criterion. Since diabetes is an important confounder for cognitive function, we further sensitivity analyzed the stepwise regression models with additional diabetes comorbidity adjustment, the results remain similar. In our subgroup analysis, the

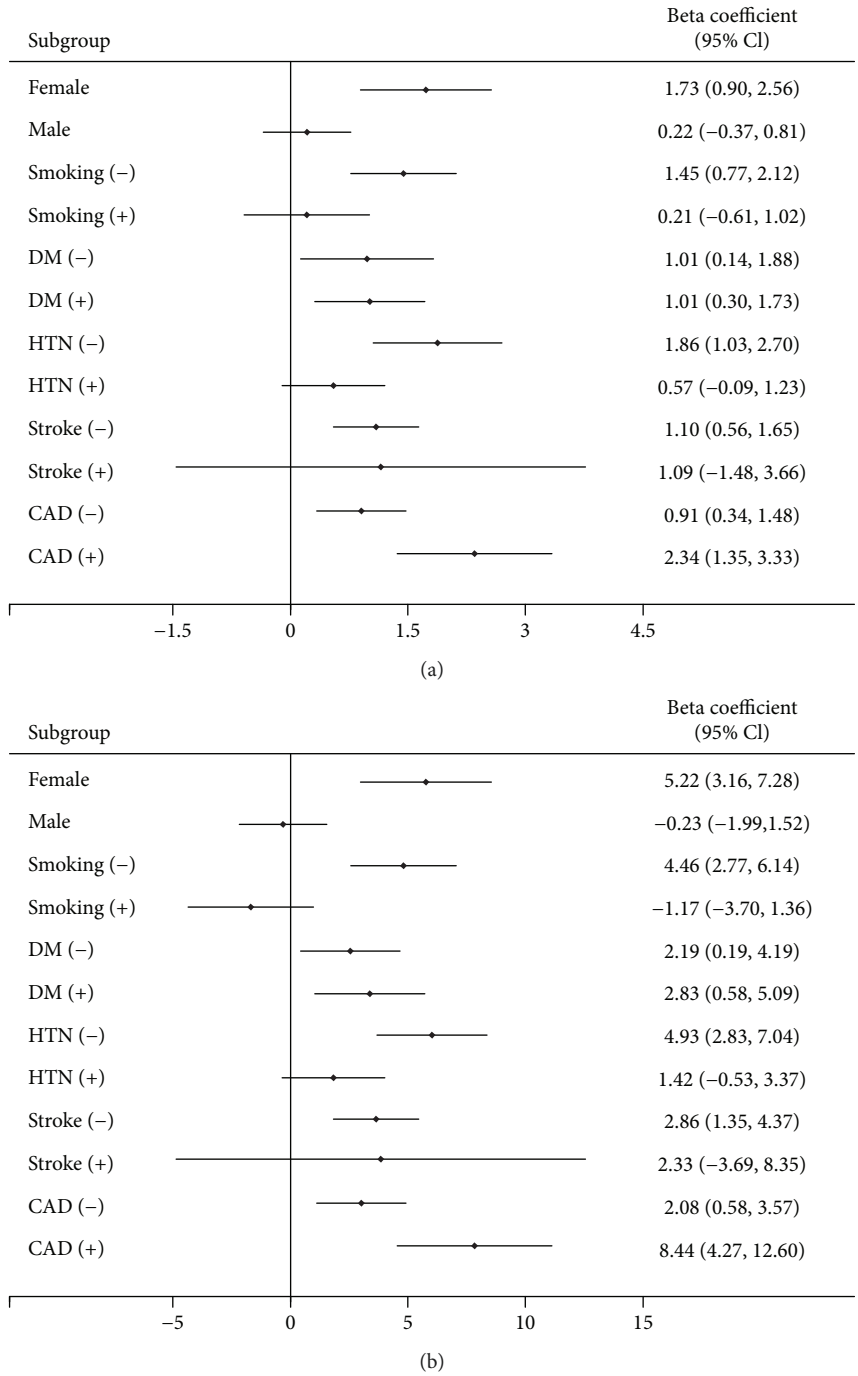

Figure 1: Subgroup analysis of the association between ABI and cognitive function test; (a) MoCA test and (b) CASI test. DM: diabetes mellitus; HTN: hypertension; CAD: coronary artery disease.

association between ABI/baPWV and cognitive function was still significant in diabetes mellitus strata. Thus, the relationship between ABI/baPWV and cognition was similar in patients with and without diabetes comorbidity.

Besides, a positive association was found between the duration of dialysis and cognitive function test (CASI) in the multivariate linear regression model. Although this association is counterintuitive, it could reflect a selection bias or survivor bias inherent in prevalent dialysis cohorts. Survivors of long-term dialysis often begin dialysis at a young age and have had no opportunity for renal transplantation. Young and healthy dialysis patients would actually have better cognitive function than older or more ill dialysis patients. In the stepwise selection model, the significant association between the duration of dialysis and the cognitive function test may be related to open a noncausal path because of nonconfounder (Collider variables) adjustment in the causal pathway between duration of dialysis and cognitive function.

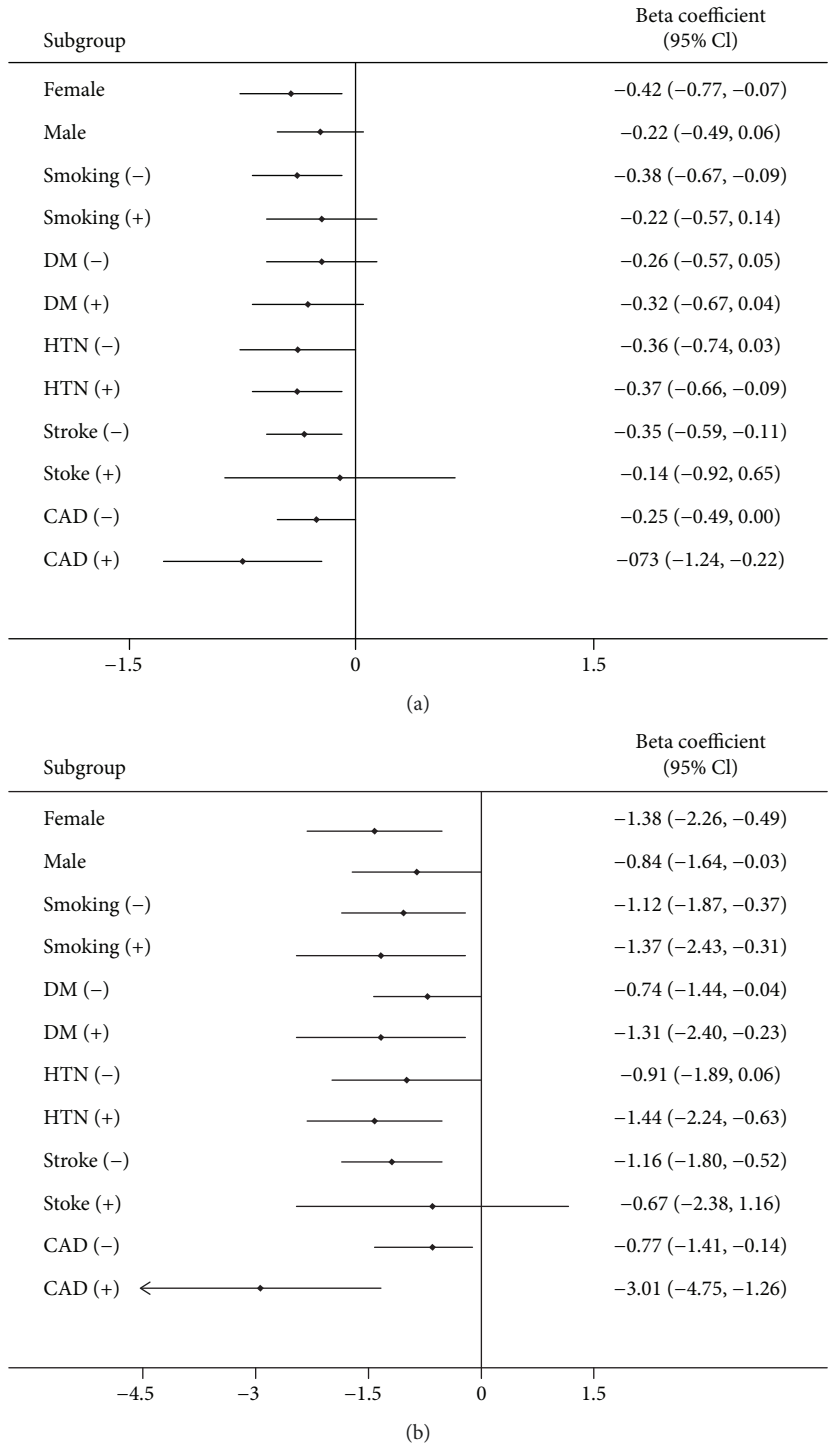

Figure 2: Subgroup analysis of the association between baPWV and cognitive function test; (a) MoCA test and (b) CASI test. DM: diabetes mellitus; HTN: hypertension; CAD: coronary artery disease.

On the contrary, our study interest is the association between ABI/baPWV and cognitive function that variable selection fits the causal pathway models in our study.

The important clinical implications of our findings are that ABI and baPWV are noninvasive and easily measured parameters that can be used to help identify the HD patients at risk of cognitive dysfunction by evaluating the degree of arterial stiffness. However, there are several limitations to this study. First, a causal relationship could not be inferred due to the cross-sectional nature of the study. Nonetheless, our results may help to identify the risk of cognitive dysfunction in HD patients through regular ABI examinations. Since a long-term decline in cognitive function could not be confirmed in this study, future prospective studies are needed

to address this issue. Second, this study was conducted at a single regional hospital, thereby limiting the selection and the number of patients. In addition, patients with peritoneal dialysis were not enrolled in this study; therefore, the results may not be applicable to patients undergoing peritoneal dialysis. Further studies are needed to confirm our findings. Third, ABI measurements and cognitive function tests were performed only once in each patient, which may have caused misclassification. Fourth, not all possible parameters were included in this study such as dietary habits, genetic factors, and medications. In addition, neuroimaging assessments could not be performed, so asymptomatic brain lesions could not be fully investigated. Moreover, different types of dementia and cognitive impairment etiology may have affected the results.

5. Conclusion

In this study, the HD patients with a low ABI and high baPWV were associated with a lower cognitive function, even after adjusting for age and hemodynamic and risk factors for atherosclerosis. Future studies should explore the longitudinal associations between ABI and baPWV and cognitive decline in a representative sample of HD patients. Our findings should serve to remind physicians of the importance of evaluating PAD in addition to cognitive function in HD patients.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Table 1: description of the neuropsychiatric test on patients with hemodialysis in the study. Supplementary Table 2: determinants of MoCA and ABI association using multivariate stepwise linear regression analysis. Supplementary Table 3: determinants of CASI and ABI association using multivariate stepwise linear regression analysis. Supplementary Table 4: determinants of MoCA and baPWV association using multivariate stepwise linear regression analysis. Supplementary Table 5: determinants of CASI and baPWV association using multivariate stepwise linear regression analysis. Supplementary Table 6: the association between comorbidities and cognitive function (MoCA and CASI). Supplementary Table 7: the association between ABI and cognitive function test (MoCA and CASI) using multivariate stepwise linear regression analysis and further additional diabetes mellitus comorbidity adjustment. Supplementary Table 8: the association between baPWV and cognitive function test (MoCA and CASI) using multivariate stepwise linear regression analysis and further additional diabetes mellitus comorbidity adjustment. *(Supplementary Materials)*

References

[1] E. O'Lone, M. Connors, P. Masson et al., "Cognition in people with end-stage kidney disease treated with hemodialysis: a systematic review and meta-analysis," *American Journal of Kidney Diseases*, vol. 67, no. 6, pp. 925–935, 2016.

[2] A. J. Collins, B. Kasiske, C. Herzog et al., "United States renal data system 2006 annual data report abstract," *American Journal of Kidney Diseases*, vol. 49, pp. A6–A7, 2007.

[3] M. Kurella, D. L. Mapes, F. K. Port, and G. M. Chertow, "Correlates and outcomes of dementia among dialysis patients: the Dialysis Outcomes and Practice Patterns Study," *Nephrology, Dialysis, Transplantation*, vol. 21, no. 9, pp. 2543–2548, 2006.

[4] D. A. Rakowski, S. Caillard, L. Y. Agodoa, and K. C. Abbott, "Dementia as a predictor of mortality in dialysis patients," *Clinical Journal of the American Society of Nephrology*, vol. 1, no. 5, pp. 1000–1005, 2006.

[5] J. M. Chillon, Z. A. Massy, and B. Stengel, "Neurological complications in chronic kidney disease patients," *Nephrology, Dialysis, Transplantation*, vol. 31, no. 10, pp. 1606–1614, 2016.

[6] A. Scuteri, A. M. Brancati, W. Gianni, A. Assisi, and M. Volpe, "Arterial stiffness is an independent risk factor for cognitive impairment in the elderly: a pilot study," *Journal of Hypertension*, vol. 23, no. 6, pp. 1211–1216, 2005.

[7] Y. Leskinen, J. P. Salenius, T. Lehtimaki, H. Huhtala, and H. Saha, "The prevalence of peripheral arterial disease and medial arterial calcification in patients with chronic renal failure: requirements for diagnostics," *American Journal of Kidney Diseases*, vol. 40, no. 3, pp. 472–479, 2002.

[8] J. R. Bartholomew and J. W. Olin, "Pathophysiology of peripheral arterial disease and risk factors for its development," *Cleveland Clinic Journal of Medicine*, vol. 73, Supplement 4, pp. S8–14, 2006.

[9] J. Blacher, M. E. Safar, A. P. Guerin, B. Pannier, S. J. Marchais, and G. M. London, "Aortic pulse wave velocity index and mortality in end-stage renal disease," *Kidney International*, vol. 63, no. 5, pp. 1852–1860, 2003.

[10] M. Guerchet, V. Aboyans, P. Nubukpo, P. Lacroix, J. P. Clement, and P. M. Preux, "Ankle-brachial index as a marker of cognitive impairment and dementia in general population. A systematic review," *Atherosclerosis*, vol. 216, no. 2, pp. 251–257, 2011.

[11] J. F. Price, S. McDowell, M. C. Whiteman, I. J. Deary, M. C. Stewart, and F. G. R. Fowkes, "Ankle brachial index as a predictor of cognitive impairment in the general population: ten-year follow-up of the Edinburgh Artery Study," *Journal of the American Geriatrics Society*, vol. 54, no. 5, pp. 763–769, 2006.

[12] D. Laurin, K. H. Masaki, L. R. White, and L. J. Launer, "Ankle-to-brachial index and dementia: the Honolulu-Asia Aging Study," *Circulation*, vol. 116, no. 20, pp. 2269–2274, 2007.

[13] D. Karasavvidou, P. Boutouyrie, R. Kalaitzidis et al., "Arterial damage and cognitive decline in chronic kidney disease patients," *Journal of Clinical Hypertension*, vol. 20, no. 9, pp. 1276–1284, 2018.

[14] A. Tasmoc, M. D. Donciu, G. Veisa, I. Nistor, and A. Covic, "Increased arterial stiffness predicts cognitive impairment in hemodialysis patients," *Hemodialysis International*, vol. 20, no. 3, pp. 463–472, 2016.

[15] H.-M. Su, J.-M. Chang, F.-H. Lin et al., "Influence of different measurement time points on brachial-ankle pulse wave velocity and ankle-brachial index in hemodialysis patients," *Hypertension Research*, vol. 30, no. 10, pp. 965–970, 2007.

[16] H. Tomiyama, A. Yamashina, T. Arai et al., "Influences of age and gender on results of noninvasive brachial-ankle pulse wave velocity measurement—a survey of 12517 subjects," *Atherosclerosis*, vol. 166, no. 2, pp. 303–309, 2003.

[17] A. S. Costa, F. E. Tiffin-Richards, B. Holschbach et al., "Clinical predictors of individual cognitive fluctuations in patients undergoing hemodialysis," *American Journal of Kidney Diseases*, vol. 64, no. 3, pp. 434–442, 2014.

[18] Y. T. Lin, P. H. Wu, H. H. Lee et al., "Indole-3 acetic acid increased risk of impaired cognitive function in patients receiving hemodialysis," *Neurotoxicology*, vol. 73, pp. 85–91, 2019.

[19] T. J. Hsieh, J. M. Chang, H. Y. Chuang et al., "End-stage renal disease: in vivo diffusion-tensor imaging of silent white matter damage," *Radiology*, vol. 252, no. 2, pp. 518–525, 2009.

[20] M. K. Tamura, B. Larive, M. L. Unruh et al., "Prevalence and correlates of cognitive impairment in hemodialysis patients: the Frequent Hemodialysis Network trials," *Clinical Journal of the American Society of Nephrology*, vol. 5, no. 8, pp. 1429–1438, 2010.

[21] J. B. Post, K. G. Morin, M. Sano, A. B. Jegede, E. Langhoff, and A. M. Spungen, "Increased presence of cognitive impairment in hemodialysis patients in the absence of neurological events," *American Journal of Nephrology*, vol. 35, no. 2, pp. 120–126, 2012.

[22] M. J. Sarnak, H. Tighiouart, T. M. Scott et al., "Frequency of and risk factors for poor cognitive performance in hemodialysis patients," *Neurology*, vol. 80, no. 5, pp. 471–480, 2013.

[23] F. E. Tiffin-Richards, A. S. Costa, B. Holschbach et al., "The Montreal Cognitive Assessment (MoCA) - a sensitive screening instrument for detecting cognitive impairment in chronic hemodialysis patients," *PLoS One*, vol. 9, no. 10, article e106700, 2014.

[24] S. H. Lee, A. Cho, Y. K. Min, Y. K. Lee, and S. Jung, "Comparison of the Montreal cognitive assessment and the mini-mental state examination as screening tests in hemodialysis patients without symptoms," *Renal Failure*, vol. 40, no. 1, pp. 323–330, 2018.

[25] Z. S. Nasreddine, N. A. Phillips, V. Bédirian et al., "The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment," *Journal of the American Geriatrics Society*, vol. 53, no. 4, pp. 695–699, 2005.

[26] E. Erken, O. Altunoren, M. E. Senel et al., "Impaired cognition in hemodialysis patients: the Montreal Cognitive Assessment (MoCA) and important clues for testing," *Clinical Nephrology*, vol. 91, no. 5, pp. 275–283, 2019.

[27] S. L. Seliger and M. J. Sarnak, "Subclinical vascular disease of the brain in dialysis patients," *American Journal of Kidney Diseases*, vol. 50, no. 1, pp. 8–10, 2007.

[28] A. M. Murray, "Cognitive impairment in the aging dialysis and chronic kidney disease populations: an occult burden," *Advances in Chronic Kidney Disease*, vol. 15, no. 2, pp. 123–132, 2008.

[29] D. G. Moodalbail, K. A. Reiser, J. A. Detre et al., "Systematic review of structural and functional neuroimaging findings in children and adults with CKD," *Clinical Journal of the American Society of Nephrology*, vol. 8, no. 8, pp. 1429–1448, 2013.

[30] D. A. Drew, B. B. Koo, R. Bhadelia et al., "White matter damage in maintenance hemodialysis patients: a diffusion tensor imaging study," *BMC Nephrology*, vol. 18, no. 1, p. 213, 2017.

[31] F. Forette, M. L. Seux, J. A. Staessen et al., "Prevention of dementia in randomised double-blind placebo-controlled Systolic Hypertension in Europe (Syst-Eur) trial," *The Lancet*, vol. 352, no. 9137, pp. 1347–1351, 1998.

[32] A. Yamashina, H. Tomiyama, K. Takeda et al., "Validity, reproducibility, and clinical significance of noninvasive brachial-ankle pulse wave velocity measurement," *Hypertension Research*, vol. 25, no. 3, pp. 359–364, 2002.

[33] S. Fishbane, S. Youn, E. J. Kowalski, and G. L. Frei, "Ankle-arm blood pressure index as a marker for atherosclerotic vascular diseases in hemodialysis patients," *American Journal of Kidney Diseases*, vol. 25, no. 1, pp. 34–39, 1995.

[34] J. Singer, J. N. Trollor, B. T. Baune, P. S. Sachdev, and E. Smith, "Arterial stiffness, the brain and cognition: a systematic review," *Ageing Research Reviews*, vol. 15, pp. 16–27, 2014.

[35] M. Silvestrini, I. Paolino, F. Vernieri et al., "Cerebral hemodynamics and cognitive performance in patients with asymptomatic carotid stenosis," *Neurology*, vol. 72, no. 12, pp. 1062–1068, 2009.

[36] A. H. E. A. van Beek, J. A. H. R. Claassen, M. G. M. O. Rikkert, and R. W. M. M. Jansen, "Cerebral autoregulation: an overview of current concepts and methodology with special focus on the elderly," *Journal of Cerebral Blood Flow and Metabolism*, vol. 28, no. 6, pp. 1071–1085, 2008.

[37] S. Hilal, M. Saini, C. S. Tan et al., "Ankle-brachial index, cognitive impairment and cerebrovascular disease in a Chinese population," *Neuroepidemiology*, vol. 42, no. 2, pp. 131–138, 2014.

[38] M. A. Shaik, N. Venketasubramanian, C. Y. Cheng et al., "Ankle brachial index, MRI markers and cognition: the Epidemiology of Dementia in Singapore study," *Atherosclerosis*, vol. 263, pp. 272–277, 2017.

[39] A. E. Roher, S. L. Tyas, C. L. Maarouf et al., "Intracranial atherosclerosis as a contributing factor to Alzheimer's disease dementia," *Alzheimer's & Dementia*, vol. 7, no. 4, pp. 436–444, 2011.

[40] T. T. van Sloten, A. D. Protogerou, R. M. A. Henry, M. T. Schram, L. J. Launer, and C. D. A. Stehouwer, "Association between arterial stiffness, cerebral small vessel disease and cognitive impairment: a systematic review and meta-analysis," *Neuroscience and Biobehavioral Reviews*, vol. 53, pp. 121–130, 2015.

[41] A. Zeki Al Hazzouri and K. Yaffe, "Arterial stiffness and cognitive function in the elderly," *Journal of Alzheimer's Disease*, vol. 42, Supplement 4, pp. S503–S514, 2014.

[42] A. Scuteri, M. Tesauro, S. Appolloni, F. Preziosi, A. M. Brancati, and M. Volpe, "Arterial stiffness as an independent predictor of longitudinal changes in cognitive function in the older individual," *Journal of Hypertension*, vol. 25, no. 5, pp. 1035–1040, 2007.

[43] M. Fukuhara, K. Matsumura, T. Ansai et al., "Prediction of cognitive function by arterial stiffness in the very elderly," *Circulation Journal*, vol. 70, no. 6, pp. 756–761, 2006.

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Research Article

Decreased Expression of Urinary Mammalian Target of Rapamycin mRNA Is Related to Chronic Renal Fibrosis in IgAN

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Background. Renal fibrosis is a common outcome of all pathological types of chronic kidney disease (CKD). However, the noninvasive detection of renal fibrosis remains a challenge. *Methods*. We collected urine samples from 154 biopsy-proven IgA nephropathy (IgAN) patients and 61 healthy controls. The expression of mTOR was measured and the correlation with renal function parameter and pathological indicators. The receiver operating characteristic (ROC) curve for the diagnosis of IgAN and renal fibrosis was calculated. *Results*. The urinary mammalian target of rapamycin (mTOR) expression was decreased in IgAN patients. The expression of mTOR was correlated with serum creatinine, blood urea nitrogen, estimated glomerular filtration rate, 24 h proteinuria, and cystatin C. Further, the urinary mTOR expression was significantly decreased in severe renal fibrosis patients compared with mild or moderate renal fibrosis patients. Urinary mTOR expression was correlated with score of tubulointerstitial fibrosis (TIF) and score of glomerular sclerosis. The ROC curve showed that mTOR can diagnose IgAN at a cut-off value of 0.930 with the sensitivity of 90.2% and specificity of 73.8% and renal fibrosis at a cut-off value of 0.301 with the sensitivity of 71.7% and specificity of 64.8%. *Conclusion*. Urinary mTOR mRNA expression was a potential biomarker for diagnosis of IgAN and renal fibrosis in IgAN patients.

1. Introduction

Chronic kidney disease (CKD) is a major public health problem worldwide and in China. The morbidity of CKD is 10.8% according to a cross-sectional survey [1]. IgA nephropathy (IgAN) is the major pathological type of CKD. The mechanism of IgAN is complicated that many factors such as abnormal IgA1 molecule [2], abnormal immune regulation [3, 4], podocyte injury [5], formation of fibrous scar in tubulointerstitium [6], hereditary factors [7], and environmental factors [8] participated in the progression of IgAN. Renal fibrosis, particularly tubulointerstitial fibrosis (TIF), is the common histological outcome of all types of CKD [9]. Accurate and early diagnosis of IgAN and renal fibrosis is important for treating and monitoring the progression of CKD.

The diagnosis of renal fibrosis, also the pathological progress, relies on renal puncture biopsy and pathological staining. Renal puncture is an invasive method which may result in bleeding and other serious complications [10]. Repeated renal biopsy is rare in the clinical practice that leads to difficult monitoring of disease progression. Classic biomarkers of CKD such as serum creatinine (Scr), blood urea nitrogen (BUN), and cystatin C (Cys-c) are incapable of accurately diagnosing renal fibrosis. Therefore, it is important to find a new noninvasive procedure and biomarkers which can diagnose renal fibrosis and pathological progression of CKD.

Recently, real-time quantitative polymerase chain reaction- (qPCR-) based urinary RNA detection has been developed for years as a novel strategy for the identification of renal kidney and CKD biomarkers [11–13]. Mammalian target of rapamycin (mTOR) is the key molecule that participated in cell proliferation, inflammation, and immunomodulation. Previous studies have indicated the relation between mTOR and CKD [14, 15]. However, if urinary mTOR RNA expression is related to renal fibrosis is unknown. Accordingly, this study was designed to determine the expression of urinary mTOR via qPCR to indicate renal fibrosis.

2. Methods

2.1. Study Design and Participants. A total of 154 biopsy-proven IgAN patients were selected from the Department of Nephrology, Yi Ji Shan Hospital, Wannan Medical College. Exclusion criteria are patients younger than 18 years old from 2017 to 2018; patients with chronic liver disease, urinary tract infection, cancer, or organ transplantation; signs or symptoms of severe complications, including cardiovascular disorder; or the use of steroids or immunosuppressive medications. Urine samples were collected 24 hours after a kidney biopsy, and clinical data were collected for all enrolled patients. Age- and gender-matched healthy volunteers ($n = 61$) from the Yi Ji Shan Hospital Health Care Center were also enrolled in the study as controls. This study was approved by the Ethical Committee of Yi Ji Shan Hospital, Wannan Medical College. Written informed consents were obtained from all subjects for the use of their urine and biopsy samples for research purposes.

2.2. Collection of Urine Samples and RNA Isolation. Whole stream early morning urine specimens were collected. Urine samples were centrifuged at 3,000 g for 30 min at 4°C. The remaining cell pellets were collected and then resuspended in 1.5 ml DEPC-treated PBS and centrifuged at 13,000 g for 5 min at 4°C. After being washed three times by diethyl pyrocarbonate- (DEPC-) treated phosphate buffer saline (PBS), the pellets were resuspended in 1.0 ml TRIzol Reagent (Ambion, Life Technologies) and stored at -80°C. Total RNA was extracted according to the manufacturer's protocol (Ambion, Life Technologies). Furthermore, the concentration and purity of RNA were assessed using the relative absorbance ratio at 260/280 in a NanoDrop 2000 (Thermo). 18S RNA was measured as the control.

2.3. Real-Time RT-qPCR. RT-PCR was performed using mTOR primers (sense: 5-TCCGAGAGATGAGTCAAGAGG-3; antisense: 5-CACCTTCCACTCCTATGAGGC-3) and 18S rRNA primers (sense: 5-CATGCTAACTAGTTACGCGACC-3; antisense: 5-GAGCAATAACAGGTCTGTGATG-3). After RT (50°C, 30 min), hot start (94°C, 15 min), and 40–42 cycles of PCR (94°C, 1 min; 52.5°C, 1 min; and 72°C, 1 min), mTOR mRNA expression was normalized to 18S rRNA and calculated as $2^{-\Delta\Delta Ct}$.

2.4. Assessment of Renal Fibrosis. Renal fibrosis was performed on paraffin-embedded sections stained with periodic acid-Schiff and Masson trichrome. Serial 3 m sections were acquired from each paraffin block. Two experienced pathologists who were blinded to the results of molecular studies subjectively scored the severity of renal fibrosis. Glomerulosclerosis was assessed in periodic acid-Schiff-stained sections using a semiquantitative scoring system according to the method of Schaier et al. [16]. Each glomerulus was graded from 0 to 4 according to sclerosis severity, and the average of all glomeruli in the entire tissue sample was calculated for analysis. The evaluation of the percentage of TIF was performed on Masson-stained sections and estimated the severity of TIF [17]. None was considered to be up to 5% of the renal interstitium, moderate between 26 and 50%, and severe > 50%. For analysis, biopsies with a TIF area < 25% were combined as none-to-mild fibrosis. Oxford histological classification was performed according to previous research [18, 19].

2.5. Statistical Analysis. SPSS 17.0 was used for data analysis. Relative changes in gene expression were calculated using the $\Delta\Delta$Ct (threshold cycle) method: ΔCt = Ct gene of interest-Ct internal control, while $\Delta\Delta$Ct = (Ct gene of interest-Ct internal control) sample-(Ct gene of interest-Ct internal control) control. Fold change = target gene expression level of sample/target gene expression level of control = $2^{-\Delta\Delta Ct}$. Normal distribution and equal variance data were compared using Student's t-test. A Mann–Whitney test was used for variance inequality or nonnormal distribution data. Spearman's rank-order correlation coefficient was used to assess associations between gene expression levels and clinical parameters. Stepwise multivariate logistic regression analysis including all univariate associates ($P < 0.05$) was used to assess the predictors for renal fibrosis. The diagnostic performance of biomarkers was evaluated using receiver operating characteristic (ROC) curves. The diagnostic threshold for maximum sensitivity and specificity was calculated. All P values were two-tailed, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline Clinical and Pathological Characteristics. Kidney biopsies were performed for the clinical diagnosis of IgAN. Primary clinical and pathological characteristics of the involved subjects are summarized in Table 1. There were no significant differences in age and gender between IgAN patients and controls. The IgAN group had a significant decrease in the estimated glomerular filtration rate (eGFR) compared with controls. eGFR was calculated using modified MDRD equations for Chinese patients [20]. Relative expression of mTOR was significantly decreased in the IgAN group ($P < 0.05$ vs. controls, Figure 1(a)). Oxford histological classification of IgAN patients was also shown in Table 1. Furthermore, the 154 IgAN patients were divided into 3 groups according to renal fibrosis degree. As shown in Table 2, there were no significant differences in age, gender, 24 h proteinuria, and BUN among the three groups. The eGFR in severe renal fibrosis was significantly lower compared with the other two groups. The relative expression of mTOR was significantly lower in moderate ($P < 0.05$ vs.

Table 1: Clinical profile of patients with IgA nephropathy and healthy controls at the time of the kidney biopsy.

	IgAN ($n = 154$)	Control ($n = 61$)	P value
Age (years)	36.7 ± 1.6[a]	35.6 ± 2.1[a]	0.097[c]
Gender (male/female)	86/68	37/24	0.157[d]
Proteinuria (g/day)	2.1 ± 0.7[a]	n.d.	—
Scr (mmol/l)	146.8 ± 35.4[a]	54.9 ± 9.8[a]	<0.001[c]
BUN (mmol/l)	7.8 ± 3.6[a]	4.2 ± 1.1[a]	<0.001[c]
Cystatin C (mg/l)	1.27 ± 0.7[a]	0.68 ± 0.2[a]	<0.001[c]
eGFR (ml/min per 1.73 m^2)	74.5 ± 8.3[a]	131.8 ± 10.2[a]	<0.001[c]
SBP (mmHg)	138.1 ± 6.4[a]	121.6 ± 7.9[a]	<0.001[c]
DBP (mmHg)	89.8 ± 7.6[a]	76.4 ± 5.6[a]	0.026[c]
Relative mTOR expression	0.412 (0.158-0.597)[b]	1.147 (0.217-1.380)[b]	<0.001[e]
Usage of ACEI/ARB	96	n.d.	—
Usage of diuretic	83	n.d.	—
Oxford histological classification (number)			
M score (0/1)	0/154	n.d.	—
E score (0/1)	80/74	n.d.	—
S score (0/1)	61/93	n.d.	—
T score (0/1/2)	62/65/27	n.d.	—
C score (0/1/2)	145/7/2	n.d.	—

[a]Data are presented as means (±SD). [b]Data are presented as the median (min, max). [c]Student's t-test. [d]χ^2 tests. [e]Mann–Whitney test. Abbreviations: Scr: serum creatinine; BUN: blood urea nitrogen; eGFR: estimated glomerular filtration rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; M: mesangial hypercellularity; E: endocapillary hypercellularity; S: segmental glomerulosclerosis; T: tubular atrophy/interstitial fibrosis; n.d.: not determined.

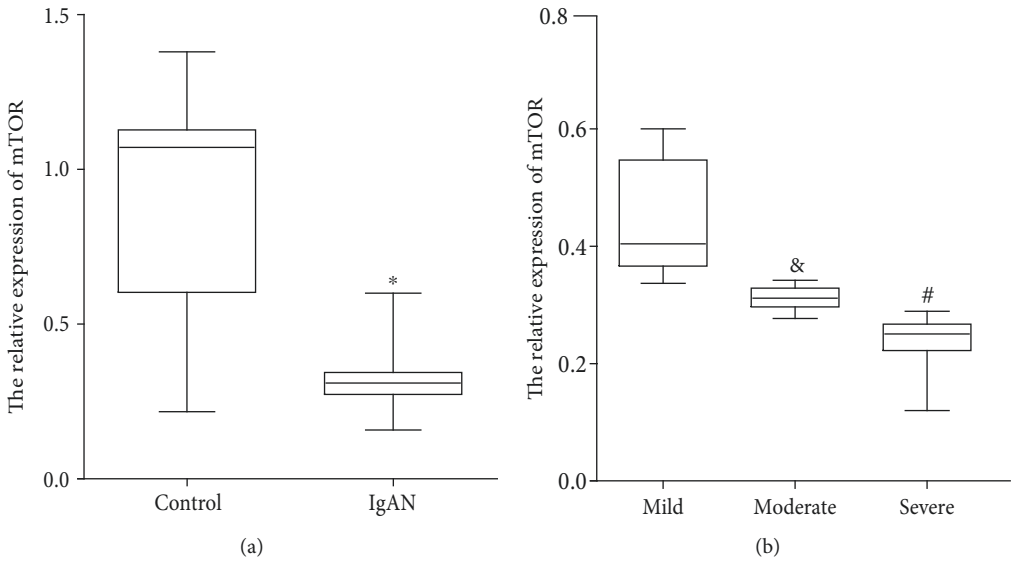

Figure 1: Urinary mTOR mRNA expression in IgAN patients and controls. (a) The relative expression of urinary mTOR in IgAN and healthy controls. (b) The relative expression of urinary mTOR in different degrees of renal fibrosis patients ($^*P < 0.05$ vs. control; $^\#P < 0.05$ vs. mild and moderate; and $^\&P < 0.05$ vs. severe).

none-mild) and severe fibrosis ($P < 0.05$ vs. moderate, none-mild) groups than none-mild fibrosis (Figure 1(b)). Figure 2 showed the representative of different degrees of renal fibrosis confirmed by Masson trichrome.

3.2. Correlation between Urinary mTOR Expression, Clinical Parameters, and Renal Fibrosis. As shown in Figure 3, urinary mTOR mRNA levels correlated with Scr ($r_s = -0.430$, $P < 0.001$), BUN ($r_s = -0.475$, $P < 0.001$), eGFR ($r_s = 0.490$, $P < 0.001$), 24 h proteinuria ($r_s = -0.213$, $P = 0.041$), and Cys-c ($r_s = -0.506$, $P < 0.001$). Further, as shown in Figure 4, urinary mTOR mRNA levels correlated with score of TIF ($r_s = -0.563$, $P < 0.001$) and score of glomerular sclerosis ($r_s = -0.552$, $P < 0.001$).

Stepwise multivariate logistic regression analysis showed that the relative expression of urinary mTOR strongly

Table 2: Clinical and pathological parameters of patients with IgAN with different renal fibrosis degrees.

	None-mild fibrosis ($n = 64$)	Moderate fibrosis ($n = 54$)	Severe fibrosis ($n = 36$)	P value
Age (years)	34.25 ± 9.13[a]	39.39 ± 16.55[a]	40.54 ± 9.38[a]	0.254[c]
Gender (male/female)	30/34	33/21	19/17	0.526[d]
Proteinuria (g/day)	1.8 ± 0.6[a]	2.1 ± 1.9[a]	2.3 ± 1.8[a]	0.125[c]
Scr (μmol/l)	60.26 ± 15.15[a]	179.28 ± 24.45[a]	187.74 ± 42.35[a]	0.021[c]
BUN (mmol/l)	8.9 ± 1.8[a]	9.2 ± 3.7[a]	10.8 ± 3.2[a]	0.074[c]
Cystatin C (mg/l)	0.75 ± 0.14[a]	1.21 ± 0.27[a]	1.53 ± 0.43[a]	0.042[c]
eGFR (ml/min per 1.73 m^2)	96.32 ± 18.78[a]	64.36 ± 23.85[a]	50.24 ± 19.24[a]	<0.001[c]
SBP (mmHg)	121.56 ± 14.71[a]	142.83 ± 20.73[a]	157.15 ± 30.69[a]	<0.001[c]
DBP (mmHg)	80.56 ± 16.39[a]	82.41 ± 20.15[a]	93.21 ± 14.24[a]	0.018[c]
Usage of ACEI/ARB	51	32	13	0.067[d]
Usage of diuretic	38	35	10	0.053[d]
Relative mTOR mRNA expression	0.427 (0.382-0.597)[b]	0.312 (0.276-0.341)[b]	0.223 (0.158-0.289)[b]	0.024[e]
Score of glomerular sclerosis	0.5 (0-0.8)[b]	1.8 (0.5-3.0)[b]	2.5 (2.0-4.8)[b]	<0.001[e]
Score of TIF (%)	5 (0-4)[b]	30 (27-51)[b]	61 (52-90)[b]	<0.001[e]

[a]Data are presented as means (±SD). [b]Data are presented as the median (min, max). [c]Student's t-test. [d]χ^2 tests. [e]Mann–Whitney test. Abbreviations: Scr: serum creatinine; eGFR: estimated glomerular filtration rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; TIF: tubulointerstitial fibrosis.

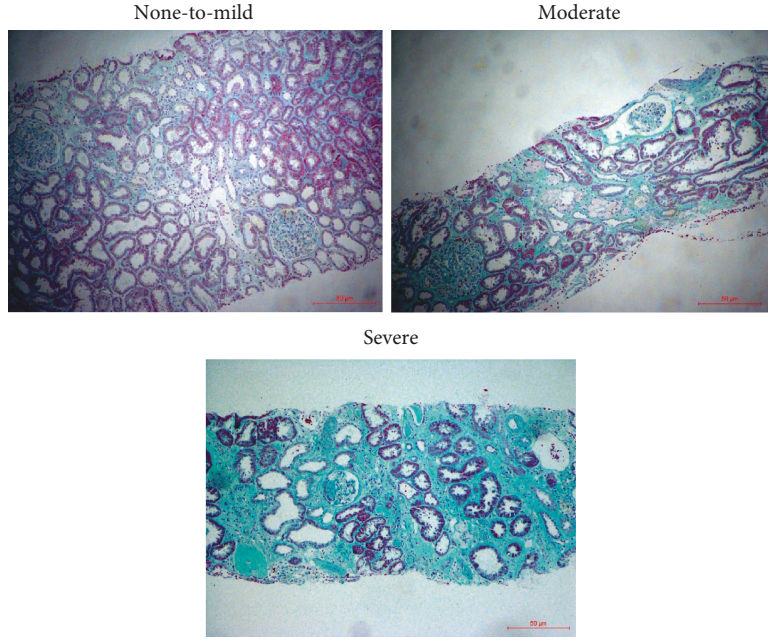

Figure 2: Representative histological findings of renal fibrosis stained by Masson's trichrome. None-to-mild fibrosis was considered as up to 25% of the tubulointerstitial fibrosis (TIF) area, moderate referred to an area 26%-50% of the TIF area, and severe referred to an area > 50% of the TIF area. Original magnification: ∗100.

correlated with the severity of renal fibrosis (Table 3, OR 10.325, 95% CI: 1.147-50.621, $P < 0.001$). The results indicated that the expression of urinary mTOR decreased every one unit; the risk for renal fibrosis elevated 10.358 times.

3.3. Diagnostic Value of Urinary mTOR mRNA Expression. The receiver operating characteristic (ROC) curve showed that the urinary mTOR mRNA level effectively distinguished IgAN from controls, with the largest AUC of 0.868 (95% CI: 0.802–0.933; $P < 0.001$), higher than that of eGFR (AUC of 0.738; 95% CI: 0.660–0.816; $P < 0.001$), Scr (AUC of 0.769; 95% CI: 0.697–0.84; $P < 0.001$), BUN (AUC of 0.618; 95% CI: 0.590–0.766; $P < 0.001$), and Cys-c (AUC of 0.704; 95% CI: 0.634-0.774; $P < 0.001$). mTOR displayed the sensitivity of 90.2% and specificity of 73.8% at the optimal cut-off value of 0.930 (relative gene expression level, Figure 5(a)).

Further, we evaluated the diagnostic value of urinary mTOR mRNA expression for renal fibrosis. The results showed that the urinary mTOR mRNA level effectively distinguished moderate-to-severe fibrosis from none-mild

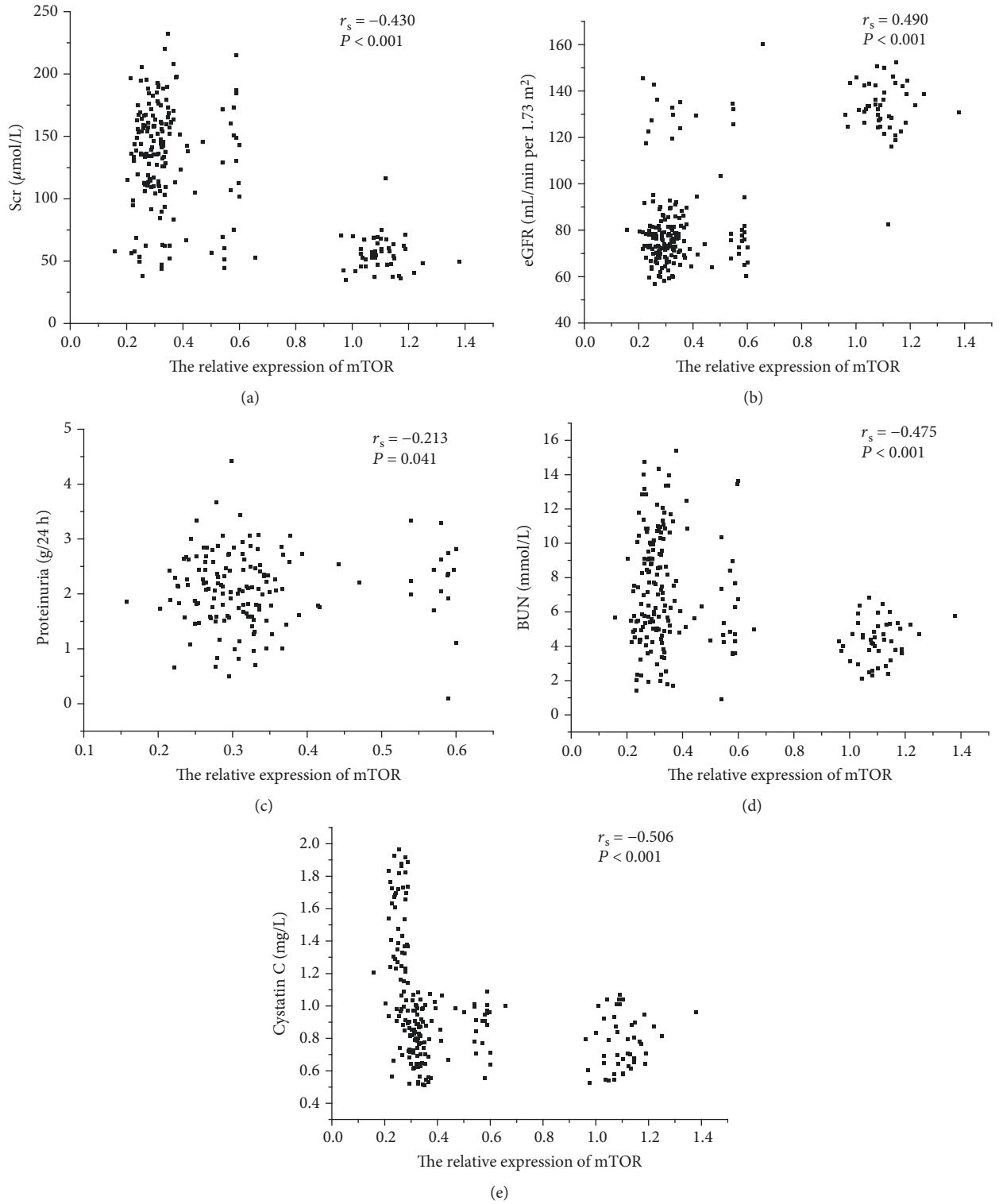

Figure 3: Correlation between urinary mTOR expression and clinical parameters. (a) Spearman correlation between mTOR expression and Scr (r_s = -0.430, $P < 0.001$). (b) Spearman correlation between mTOR expression and eGFR ($r_s = 0.490$, $P < 0.001$). (c) Spearman correlation between mTOR expression and 24 h proteinuria (r_s = -0.213, $P = 0.041$). (d) Spearman correlation between mTOR expression and BUN (r_s = -0.475, $P < 0.001$). (e) Spearman correlation between mTOR expression and cystatin C (r_s = -0.506, $P < 0.001$).

fibrosis, with the largest AUC of 0.739 (95% CI: 0.654-0.824; $P < 0.001$), higher than that of eGFR (AUC of 0.492; 95% CI: 0.395-0.588; $P = 0.868$), Scr (AUC of 0.513; 95% CI: 0.414-0.612; $P = 0.794$), BUN (AUC of 0.499; 95% CI: 0.400-0.598; $P = 0.981$), 24 h proteinuria (AUC of 0.510; 95% CI: 0.401-0.618; $P = 0.850$), and Cys-c (AUC of 0.495; 95% CI:

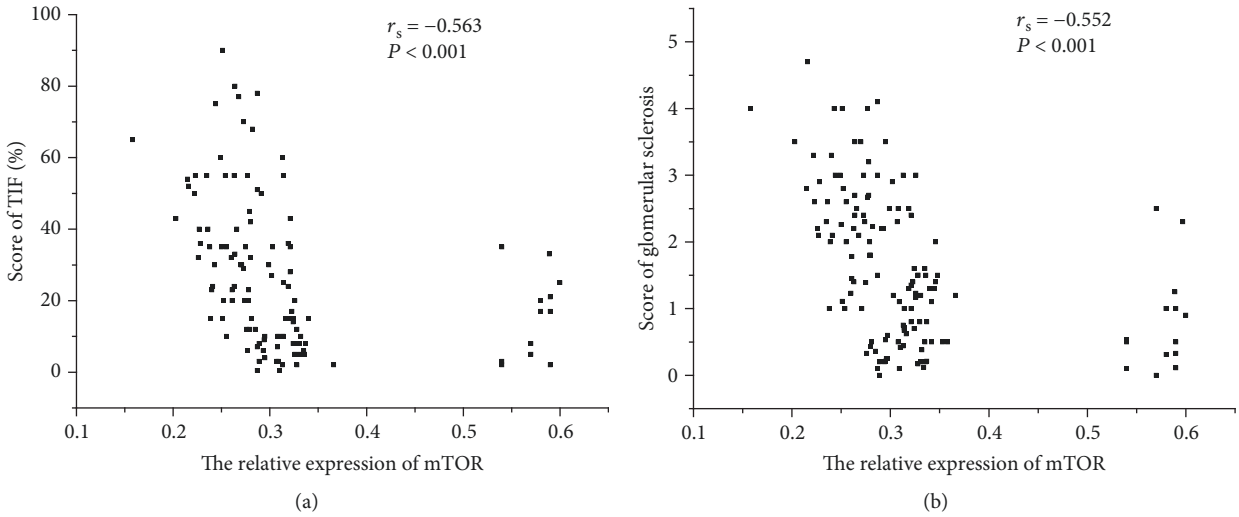

Figure 4: Correlation between urinary mTOR expression and renal fibrosis. (a) Spearman correlation between mTOR expression and score of TIF (r_s = -0.563, $P < 0.001$). (b) Spearman correlation between mTOR expression and score of glomerular sclerosis (r_s = -0.552, $P < 0.001$).

Table 3: Multivariate logistic regression analysis of selected variables for TIF severity.

	OR	95% CI	*P* value
mTOR	10.358	1.147-50.621	<0.001
Scr	0.513	0.245-2.381	0.225
BUN	0.425	0.112-3.651	0.307
Cystatin C	0.624	0.159-4.215	0.521
eGFR	1.834	1.025-4.021	0.031
24 h proteinuria	1.657	0.567-3.441	0.104

Abbreviations: Scr: serum creatinine; eGFR: estimated glomerular filtration rate; OR: odds ratio.

0.388-0.603; $P = 0.055$). mTOR displayed the sensitivity of 71.7% and specificity of 64.8% at the optimal cut-off value of 0.301 (relative gene expression level, Figure 5(b)).

ROC curves for distinguishing E1 from E0, S1 from S0, and T2 from T0 and T1 were also performed. The results showed that the urinary mTOR mRNA level effectively distinguished E1 from E0 (Figure 6(a)), with the largest AUC of 0.841 (95% CI: 0.770-0.912; $P < 0.001$), higher than that of eGFR (AUC of 0.551; 95% CI: 0.457-0.644; $P = 0.281$), Scr (AUC of 0.476; 95% CI: 0.385-0.568; $P = 0.617$), BUN (AUC of 0.488; 95% CI: 0.396-0.580; $P = 0.799$), 24 h proteinuria (AUC of 0.707; 95% CI: 0.610-0.804; $P = 0.051$), and Cys-c (AUC of 0.362; 95% CI: 0.273-0.450; $P = 0.063$). mTOR displayed the sensitivity of 91.2% and specificity of 86.0% at the optimal cut-off value of 0.308. For S score (Figure 6(b)), the results showed that the urinary mTOR mRNA level effectively distinguished S1 from S0, with the largest AUC of 0.881 (95% CI: 0.823-0.940; $P < 0.001$), higher than that of eGFR (AUC of 0.476; 95% CI: 0.384-0.568; $P = 0.614$), Scr (AUC of 0.520; 95% CI: 0.425-0.614; $P = 0.682$), BUN (AUC of 0.462; 95% CI: 0.367-0.556; $P = 0.424$), 24 h proteinuria (AUC of 0.772; 95% CI: 0.689-0.884; $P = 0.070$), and Cys-c (AUC of 0.437; 95% CI: 0.343-0.530; $P = 0.184$). mTOR displayed the sensitivity of 82.8% and specificity of 90.7% at the optimal cut-off value of 0.312. For T score (Figure 6(c)), the urinary mTOR mRNA level effectively distinguished T2 from T0 and T1, with the largest AUC of 0.909 (95% CI: 0.857-0.961; $P < 0.001$), higher than that of eGFR (AUC of 0.468; 95% CI: 0.376-0.560; $P = 0.505$), Scr (AUC of 0.497; 95% CI: 0.401-0.594; $P = 0.954$), BUN (AUC of 0.516; 95% CI: 0.420-0.611; $P = 0.740$), 24 h proteinuria (AUC of 0.757; 95% CI: 0.655-0.860; $P = 0.054$), and Cys-c (AUC of 0.485; 95% CI: 0.391-0.578; $P = 0.068$). mTOR displayed the sensitivity of 82.6% and specificity of 90.2% at the optimal cut-off value of 0.313.

4. Discussion

Our study firstly indicated that urinary mTOR mRNA was a potential noninvasive biomarker of IgAN and especially chronic renal fibrosis. The discovery of CKD and renal fibrosis is a challenging topic attracting several of researchers' direction. The traditional biomarkers of CKD, for example, Scr, BUN, and eGFR, failed to reveal the pathological type of CKD. So far, there was a lack of reliable biomarkers of renal fibrosis, although many studies suggested some candidates [21, 22]. IgAN is a major pathological type of CKD. The previous study indicated that renal CD147 expression is a potential biomarker for IgAN [23]. Other researchers further screened tissue-specific microRNA expression in glomeruli and proximal tubules in IgAN patients [24]. However, renal biopsy was also the unique method of collecting kidney tissue which limited the application in the clinical practice. Repeated renal biopsy has a high risk level for patients. So far, there was no noninvasive method to identify the severity of renal fibrosis.

The urine contained an abundant biological message which can reflect the pathological changes. The changes of some renal fibrosis-associated molecule may reflect in urinary sediment cells, especially the podocyte and tubular

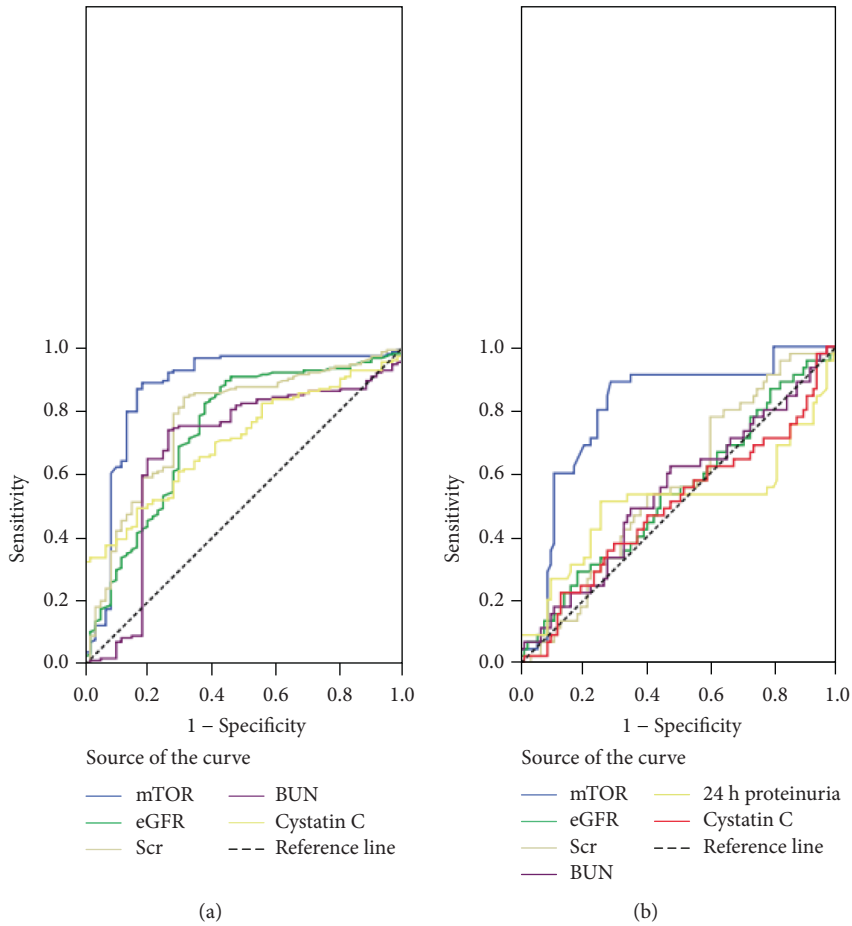

Figure 5: Receiver operating characteristic (ROC) curve showed the diagnosis value of urinary mTOR expression for IgAN and renal fibrosis. (a) ROC curve showed that the urinary mTOR mRNA level distinguished IgAN from controls (AUC = 0.868; 95% CI: 0.802-0.933; $P < 0.001$). (b) ROC curve showed that the urinary mTOR mRNA level distinguished moderate-to-severe fibrosis from none-mild fibrosis (AUC = 0.739; 95% CI: 0.654-0.824; $P < 0.001$).

epithelial cells. In recent years, qPCR was applicable in urinary mRNA biomarker detection. Li et al. [11] firstly established a noninvasive procedure to diagnose acute renal rejection of allografts by isolating and quantifying RNA of specific genes in urine cells. The obvious advance of qPCR such as high sensitivity and good repeatability makes qPCR a suitable noninvasive method to reflect kidney disease. Urinary sediment cell analysis was an appropriate procedure to screen novel mRNA biomarkers. Many previous studies indicated that urinary mRNA was a potential biomarker candidate of CKD [13, 25–27]. Zhou et al. further suggested that urinary mRNA expression showed favorable performance in diagnosing early renal fibrosis [28]. Our research revealed that urinary mRNA detection via qRT-PCR was a feasible method to identify the new biomarkers for IgAN and renal fibrosis. Urinary sediment was an appropriate source for IgAN and renal fibrosis diagnosis.

mTOR was a widely studied molecule which can regulate cell proliferation, inflammation reaction, and immune reaction [29, 30]. mTOR also participated in renal disease progression. Zhang et al. reported that mTOR signal pathways regulated immunosuppressive function in acute kidney disease [31]. In renal disease, mTOR has been identified as a potential therapy target [32]. Previous studies also indicated that mTOR played a key role in renal fibrosis [33, 34]. However, in different organs, microenvironments, and study objects, the role of mTOR showed an obvious heterogeneity. The key role of mTOR may act to adjust the balance of proinflammatory and anti-inflammatory responses [29]. In this study, we found that in IgAN patients' urinary sediment cells, mTOR mRNA expression was downregulated. Urinary sediment cells contain various types of cells such as podocyte, tubular epithelial cells, collecting duct cells, and even urethral epithelial cells. The downregulation of urinary mTOR mRNA may be a protective reaction in pathological status. The urinary mTOR expression level has diagnosis value for IgAN and renal fibrosis.

The previous studies showed that mTOR was considered as a biomarker of cancer, immune disease, degenerative diseases, and metabolic diseases [35, 36]. In IgAN, inhibition of mTOR ameliorates kidney injury [37]. It remained unknown if mTOR can act as a biomarker for IgAN, especially for renal fibrosis. Our study revealed that urinary mTOR mRNA expression was a potential biomarker for

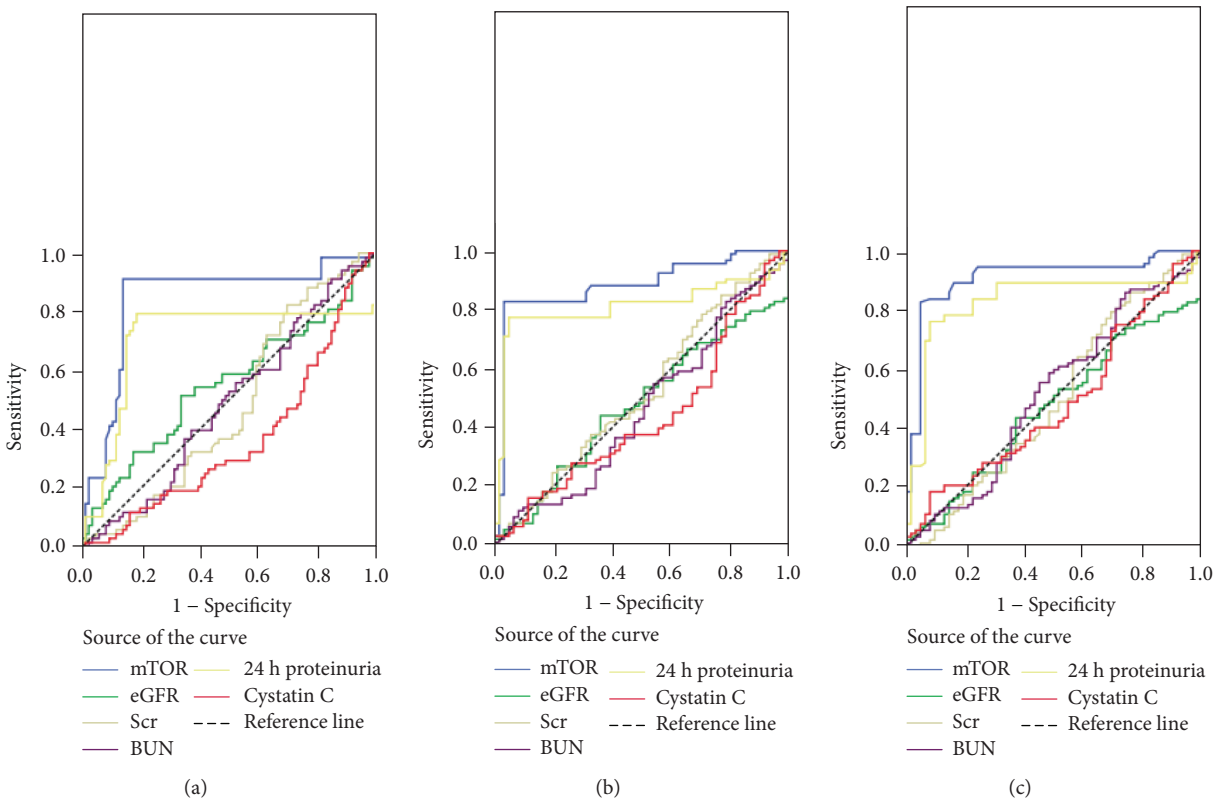

Figure 6: Receiver operating characteristic (ROC) curve showed the diagnosis value of urinary mTOR expression for E, S, and T scores. (a) ROC curve showed that the urinary mTOR mRNA level distinguished E1 from E0 (AUC = 0.841; 95% CI: 0.770-0.912; $P < 0.001$). (b) ROC curve showed that the urinary mTOR mRNA level distinguished S1 from S0 (AUC = 0.881; 95% CI: 0.823-0.940; $P < 0.001$). (c) ROC curve showed that the urinary mTOR mRNA level distinguished T2 from T0 and T1 (AUC = 0.909; 95% CI: 0.857-0.961; $P < 0.001$).

IgAN and renal fibrosis. Additionally, urinary mTOR mRNA expression also correlated with renal fibrosis in IgAN. Moreover, the ROC curve showed that urinary mTOR mRNA expression has potential to diagnose mesangial hypercellularity and endocapillary cellularity. A previous study indicated that the mTOR pathway has an important pathogenic role in diabetic nephropathy. However, if the mTOR signal pathway participated in the glomerular hypertrophy, then renal hyperplasia was uncertain [38]. Our research indicated that urinary mTOR mRNA can serve as a potential biomarker to diagnose renal fibrosis. It may be a potential noninvasive procedure that can identify IgAN and renal fibrosis.

In summary, our study demonstrated that detection of urinary mTOR mRNA could well predict renal fibrosis severity in IgAN, which suggested that this will serve as a novel independent noninvasive biomarker to monitor the progression of kidney fibrosis in IgAN.

Our study also has some limitations. Firstly, the current study is a discovery study focused on IgAN; if urinary mTOR expression can serve as a biomarker of renal fibrosis in other types of CKD needs to be further studied. Secondly, urethral epithelial cells in urinary sediment may influence the reliability of this method. Separation of different types of kidney cells can improve accuracy. Thirdly, to confirm the diagnostic value of urine mTOR mRNA in renal fibrosis and even the mesangial hypercellularity and endocapillary cellularity, a larger group of validation study and a long-term follow-up study are also necessary.

5. Conclusion

Urinary mTOR mRNA detection served as a noninvasive detection of IgAN and renal fibrosis. Urinary mTOR mRNA expression was a potential biomarker for diagnosis of IgAN and renal fibrosis in IgAN patients.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] L. Zhang, F. Wang, L. Wang et al., "Prevalence of chronic kidney disease in China: a cross-sectional survey," *The Lancet*, vol. 379, no. 9818, pp. 815–822, 2012.

[2] R. J. Wyatt and B. A. Julian, "IgA nephropathy," *The New England Journal of Medicine*, vol. 368, no. 25, pp. 2402–2414, 2013.

[3] H. Suzuki, Y. Suzuki, I. Narita et al., "Toll-like receptor 9 affects severity of IgA nephropathy," *Journal of the American Society of Nephrology*, vol. 19, no. 12, pp. 2384–2395, 2008.

[4] R. Coppo, R. Camilla, A. Alfarano et al., "Upregulation of the immunoproteasome in peripheral blood mononuclear cells of patients with IgA nephropathy," *Kidney International*, vol. 75, no. 5, pp. 536–541, 2009.

[5] A. Fukuda, Y. Sato, T. Iwakiri et al., "Urine podocyte mRNAs mark disease activity in IgA nephropathy," *Nephrology, Dialysis, Transplantation*, vol. 30, no. 7, pp. 1140–1150, 2015.

[6] M. Salvadori and G. Rosso, "Update on immunoglobulin a nephropathy, part I: pathophysiology," *World Journal of Nephrology*, vol. 4, no. 4, pp. 455–467, 2015.

[7] L. Zhu, Y. L. Zhai, F. M. Wang et al., "Variants in complement factor H and complement factor H-related protein genes, CFHR3 and CFHR1, affect complement activation in IgA nephropathy," *Journal of the American Society of Nephrology*, vol. 26, no. 5, pp. 1195–1204, 2015.

[8] F. Eitner and J. Floege, "In search of a better understanding of IgA nephropathy-associated hematuria," *Kidney International*, vol. 82, no. 5, pp. 513–515, 2012.

[9] Y. Liu, "Cellular and molecular mechanisms of renal fibrosis," *Nature Reviews Nephrology*, vol. 7, no. 12, pp. 684–696, 2011.

[10] L. Wickman, F. Afshinnia, S. Q. Wang et al., "Urine podocyte mRNAs, proteinuria, and progression in human glomerular diseases," *Journal of the American Society of Nephrology*, vol. 24, no. 12, pp. 2081–2095, 2013.

[11] B. Li, C. Hartono, R. Ding et al., "Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine," *The New England Journal of Medicine*, vol. 344, no. 13, pp. 947–954, 2001.

[12] M. Suthanthiran and T. Muthukumar, "Urinary-cell mRNA and acute kidney-transplant rejection," *The New England Journal of Medicine*, vol. 369, no. 19, pp. 1858–1861, 2013.

[13] Y. H. Cao, L. L. Lv, X. Zhang et al., "Urinary vimentin mRNA as a potential novel biomarker of renal fibrosis," *American Journal of Physiology. Renal Physiology*, vol. 309, no. 6, pp. F514–F522, 2015.

[14] H. Chen, J. Zhu, Y. Liu et al., "Lipopolysaccharide induces chronic kidney injury and fibrosis through activation of mTOR signaling in macrophages," *American Journal of Nephrology*, vol. 42, no. 4, pp. 305–317, 2015.

[15] T. B. Huber, G. Walz, and E. W. Kuehn, "mTOR and rapamycin in the kidney: signaling and therapeutic implications beyond immunosuppression," *Kidney International*, vol. 79, no. 5, pp. 502–511, 2011.

[16] M. Schaier, S. Vorwalder, C. Sommerer et al., "Role of FTY720 on M1 and M2 macrophages, lymphocytes, and chemokines in 5/6 nephrectomized rats," *American Journal of Physiology Renal Physiology*, vol. 297, no. 3, pp. F769–F780, 2009.

[17] W. S. An, H. J. Kim, K. H. Cho, and N. D. Vaziri, "Omega-3 fatty acid supplementation attenuates oxidative stress, inflammation, and tubulointerstitial fibrosis in the remnant kidney," *American Journal of Physiology. Renal Physiology*, vol. 297, no. 4, pp. F895–F903, 2009.

[18] A Working Group of the International IgA Nephropathy Network and the Renal Pathology Society, I. S. D. Roberts, H. T. Cook et al., "The Oxford classification of IgA nephropathy: pathology definitions, correlations, and reproducibility," *Kidney International*, vol. 76, no. 5, pp. 546–556, 2009.

[19] H. Trimarchi, J. Barratt, D. C. Cattran et al., "Oxford Classification of IgA nephropathy 2016: an update from the IgA Nephropathy Classification Working Group," *Kidney International*, vol. 91, no. 5, pp. 1014–1021, 2017.

[20] Y. C. Ma, L. Zuo, J. H. Chen et al., "Modified glomerular filtration rate estimating equation for Chinese patients with chronic kidney disease," *Journal of the American Society of Nephrology*, vol. 17, no. 10, pp. 2937–2944, 2006.

[21] J. Rysz, A. Gluba-Brzozka, B. Franczyk, Z. Jablonowski, and A. Cialkowska-Rysz, "Novel biomarkers in the diagnosis of chronic kidney disease and the prediction of its outcome," *International Journal of Molecular Sciences*, vol. 18, no. 8, p. 1702, 2017.

[22] B. Caplin and D. Nitsch, "Urinary biomarkers of tubular injury in chronic kidney disease," *Kidney International*, vol. 91, no. 1, pp. 21–23, 2017.

[23] S. Sun, A. Zhao, R. Li et al., "CD147 renal expression as a biomarker for progressive IgAN," *Journal of Nephrology*, vol. 28, no. 3, pp. 307–314, 2015.

[24] M. A. Baker, S. J. Davis, P. Liu et al., "Tissue-specific microRNA expression patterns in four types of kidney disease," *Journal of the American Society of Nephrology*, vol. 28, no. 10, pp. 2985–2992, 2017.

[25] M. Zheng, L. L. Lv, Y. H. Cao et al., "A pilot trial assessing urinary gene expression profiling with an mRNA array for diabetic nephropathy," *PLoS One*, vol. 7, no. 5, article e34824, 2012.

[26] M. Zheng, L. L. Lv, Y. H. Cao et al., "Urinary mRNA markers of epithelial-mesenchymal transition correlate with progression of diabetic nephropathy," *Clinical Endocrinology*, vol. 76, no. 5, pp. 657–664, 2012.

[27] M. Zheng, L. L. Lv, J. Ni et al., "Urinary podocyte-associated mRNA profile in various stages of diabetic nephropathy," *PLoS One*, vol. 6, no. 5, article e20431, 2011.

[28] L.-T. Zhou, Y.-H. Cao, L.-L. Lv et al., "Feature selection and classification of urinary mRNA microarray data by iterative random forest to diagnose renal fibrosis: a two-stage study," *Scientific Reports*, vol. 7, no. 1, article 39832, 2017.

[29] S. P. Cobbold, "The mTOR pathway and integrating immune regulation," *Immunology*, vol. 140, no. 4, pp. 391–398, 2013.

[30] J. S. L. Yu and W. Cui, "Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination," *Development*, vol. 143, no. 17, pp. 3050–3060, 2016.

[31] C. Zhang, S. Wang, J. Li et al., "The mTOR signal regulates myeloid-derived suppressor cells differentiation and

immunosuppressive function in acute kidney injury," *Cell Death & Disease*, vol. 8, no. 3, p. e2695, 2017.

[32] A. Kaur and S. Sharma, "Mammalian target of rapamycin (mTOR) as a potential therapeutic target in various diseases," *Inflammopharmacology*, vol. 25, no. 3, pp. 293–312, 2017.

[33] Y. Xu, Y. Ling, F. Yang et al., "The mTOR/p70S6K1 signaling pathway in renal fibrosis of children with immunoglobulin a nephropathy," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 18, no. 3, 2017.

[34] W. Eberhardt, U. Nasrullah, and J. Pfeilschifter, "Activation of renal profibrotic TGFβ controlled signaling cascades by calcineurin and mTOR inhibitors," *Cellular Signalling*, vol. 52, pp. 1–11, 2018.

[35] A. Perl, "mTOR activation is a biomarker and a central pathway to autoimmune disorders, cancer, obesity, and aging," *Annals of the New York Academy of Sciences*, vol. 1346, no. 1, pp. 33–44, 2015.

[36] H. Lee, "Phosphorylated mTOR expression profiles in human normal and carcinoma tissues," *Disease Markers*, vol. 2017, Article ID 1397063, 8 pages, 2017.

[37] J. Tian, Y. Wang, X. Liu, X. Zhou, and R. Li, "Rapamycin ameliorates IgA nephropathy via cell cycle-dependent mechanisms," *Experimental Biology and Medicine (Maywood, N.J.)*, vol. 240, no. 7, pp. 936–945, 2015.

[38] N. Lloberas, J. M. Cruzado, M. Franquesa et al., "Mammalian target of rapamycin pathway blockade slows progression of diabetic kidney disease in rats," *Journal of the American Society of Nephrology*, vol. 17, no. 5, pp. 1395–1404, 2006.

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Research Article

Urine 5MedC, a Marker of DNA Methylation, in the Progression of Chronic Kidney Disease

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Background. Alterations in DNA methylation may be involved in disease progression in patients with chronic kidney disease (CKD). Recent studies have suggested that 5-methyl-2′-deoxycytidine (5MedC) may be a marker of hypermethylation of DNA. Currently, there is no information available regarding the urine levels of 5MedC and its association with the progression of CKD. *Method*. We examined the urine levels of 5MedC in spot urine samples from 308 patients with CKD (median age: 56 years, male: 53.2%, and glomerulonephritis: 51.0%) using a competitive enzyme-linked immunosorbent assay and investigated the relationships among urine 5MedC, urine albumin, urine α1-microglobulin (α1MG), and the laboratory parameters associated with CKD. The patients were followed for three years to evaluate renal endpoints in a prospective manner. *Results*. The urine 5MedC level was significantly increased in the later stages of CKD compared to the early to middle stages of CKD. In multiple logistic regression models, urine 5MedC was significantly associated with the prediction of later CKD stages. Urine 5MedC (median value, 65.9 μmol/gCr) was significantly able to predict a 30% decline in the estimated GFR or a development of end-stage renal disease when combined with macroalbuminuria or an increased level of urine α1MG (median value, 5.7 mg/gCr). *Conclusion*. The present data demonstrate that the urine 5MedC level is associated with a reduced renal function and can serve as a novel and potent biomarker for predicting the renal outcome in CKD patients. Further studies will be necessary to elucidate the role of urine DNA methylation in the progression of CKD.

1. Introduction

Epigenetic changes are stable and heritable but reversible modifications, including DNA methylation, posttranscriptional modifications of histone, and remodeling of chromatin [1, 2]. Among them, DNA methylation is a crucial epigenetic alteration observed in eukaryotic organisms and has been shown to be associated with many biological and cellular processes, such as embryonic development, transcription, structure of chromatin, and stability of chromosome [3, 4]. Recently, several human diseases have been reported to be associated with abnormal DNA methylation [4, 5].

Chronic kidney disease (CKD) is a significant concern given the increasing number of such patients throughout

the entire world [6]. CKD is characterized by either or both a glomerular filtration rate (GFR) less than 60 mL/min/1.73 m^2 and signs of kidney injury of at least 3 months' duration [6, 7]. A reduced estimated GFR (eGFR) and severe degree of albuminuria independently predict end-stage renal disease and mortality in CKD patients [8]. There is an urgent need to identify novel biomarkers in patients with CKD in order to better detect those at high risk of a rapid decline in the renal function so that effective therapies can be used to inhibit the disease progression [9].

A recent large-scale genome-wide evaluation of DNA methylation showed that DNA hypomethylation and hypermethylation were present at different loci in patients with CKD [10]. DNA hypermethylation in the peripheral blood may be linked to inflammation possibly associated with bacterial infections in CKD patients with incident dialysis [11].

5-Methyl-2′-deoxycytidine (5MedC) (PubChem CID: 440055) is a product of the base excision repair (BER) and nucleotide excision repair (NER) pathways of active DNA methylation. 5MedC was detected in the urine of healthy individuals as well as in those with certain diseases via several methods [12–16]; however, little is known about its levels in the urine of CKD patients or its association with progression of disease in such patients.

We therefore determined the urine levels of 5MedC and its association with progression and renal outcome in patients with CKD.

2. Methods

2.1. Study Design. The study subjects were outpatients who had visited the Division of Nephrology in Okayama University Hospital between February 2009 and February 2012. All patients were diagnosed with CKD in accordance with the eGFR and the presence of kidney damage, as characterized by the National Kidney Foundation K/DOQI Guideline [6]. The eGFR was calculated as described previously [17]. Hypertension was defined as described previously [18, 19]. The mean blood pressure (MBP) was calculated as diastolic blood pressure + (systolic blood pressure − diastolic pressure)/3.

All procedures in the current study were performed according to national and institutional ethical guidelines of human studies and guidelines in the Declaration of Helsinki. The ethics committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences approved the study (KEN1607-010). All subjects gave written informed consent.

A prospective and longitudinal study was performed to investigate the relationship among urine 5MedC levels, clinical parameters, and the renal outcome in CKD patients. The patients who participated in this study were recruited between February 2009 and February 2012. Patients were followed for up to 3 years, but those who were followed for less than 3 months ($n = 51$) or who started renal replacement therapy within 3 months of the participation ($n = 5$) were excluded. As a result, a total of 308 patients were included in the analysis. Of these patients, 199 patients (male 107, female 92) were overlapped with the previous study [19]. In accordance with the established protocol, we excluded any patients with established atherosclerotic complications (congestive heart failure, coronary artery disease, or peripheral vascular disease) [19]. Patients with infection, acute kidney injury, cancer, and Alzheimer's disease at entry were also excluded.

2.2. Laboratory Measurement of Urine Biomarkers. Spot urine samples were collected from patients in the morning, as described previously [19]. The urine 5MedC levels were measured using a Global DNA Methylation Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Cell Biolabs Inc., San Diego, CA, USA), which was a competitive enzyme immunoassay developed for the rapid quantitation and detection of 5MedC in urine directly. The quantity of 5MedC in an unknown sample is calculated by comparing its absorbance with that of a known 5MedC standard curve. The kit has a 5MedC detection sensitivity range of 150 nM to 10 μM. The concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in urine samples was also determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA) as previously described [20, 21].

The median duration of storage between collection of urine and measurement of biomarkers was 48 months (interquartile range, 34-49 months). The urine levels of albumin, total protein, creatinine (Cr), and alpha1-microglobulin (α1MG) were measured by standard methods and used to calculate the urine albumin-to-Cr ratio (urinary albumin excretion (UAE)), urine α1MG-to-Cr ratio, and urine 5MedC-to-Cr ratio.

2.3. Data Collection. Each subject's age, gender, cause of CKD, complication of diabetes mellitus, medication with antihypertensive drugs (angiotensin receptor blocker (ARB), angiotensin-converting enzyme inhibitor (ACEI), calcium channel blocker (CCB)), mean blood pressure (MBP), and other clinical laboratory data were collected. The serum creatinine concentration was measured by the enzymatic colorimetric method using an automated analyzer (JCA-BM8040; JEOL, Tokyo, Japan), as described previously [19].

2.4. Evaluation of Outcome. The primary outcome was CKD progression, defined as a composite endpoint of incident end-stage renal disease (recipient of maintenance dialysis or renal transplant) or a 30% decline in the eGFR [22, 23]. Patients were prospectively followed for a median of 36 months (interquartile range, 26–37 months). They were followed by a review of the medical record at least twice a year until December 31, 2014. Loss to follow-up and death were considered censoring events, as described previously [19].

2.5. Statistical Analyses. All values were indicated as the median (interquartile range) or number (percentage). Differences between groups were compared using Wilcoxon's test, a t-test, or log-rank test. Kaplan-Meier analyses were applied to assess the effect of urine 5MedC levels on the renal endpoint using a generalized Wilcoxon test [19, 21]. A multiple regression analysis was used to evaluate the predictors of a low eGFR using the odds ratio (OR) or adjusted OR after adjusting for relevant factors [18, 19, 21]. A P value

Table 1: Baseline characteristics of the study subjects divided by CKD stages.

	All patients	Early to Mid-CKD (stages 1 to 3)	Later CKD (stages 4 and 5)	*P* value
N	308	241	67	
Age (years)	56 (37-67)	52 (35-65)	62 (55-71)	<0.0001
Gender, male, n (%)	164 (53.2)	123 (51.0)	41 (61.2)	0.139
eGFR (ml/min/1.73 m^2)	55.4 (32.0-79.6)	63.8 (47.9-85.8)	18.6 (14.2-24.3)	<0.0001
UAE (mg/gCr)	158 (20-762)	89 (12-542)	705 (126-1431)	<0.0001
uα1MG (mg/gCr)	5.7 (2.1-14.1)	3.9 (1.7-8.5)	23.5 (11.8-48.7)	<0.0001
u5MedC (μmol/gCr)	65.9 (40.8-130.3)	59.7 (39.0-116.5)	88.3 (48.5-153.9)	0.025
Hemoglobin (g/L)	130 (116-142)	133 (123-146)	112 (101-129)	<0.0001
MBP (mmHg)	91 (84-99)	91 (83-99)	96 (85-103)	0.013
Cause of CKD, n (%)				<0.0001
Chronic GN*	157 (51.0)	146 (60.6)	11 (16.4)	
Nephrosclerosis	40 (13.0)	19 (7.9)	21 (31.3)	
Diabetic nephropathy	11 (3.6)	7 (2.9)	4 (6.0)	
Others**	100 (32.5)	69 (28.6)	31 (46.3)	
Diabetes mellitus, n (%)	36 (11.7)	27 (11.2)	9 (13.4)	0.615
Current medication, n (%)				
ARBs/ACEIs	196 (63.0)	137 (56.9)	59 (88.1)	<0.0001
CCBs	117 (38.0)	72 (29.9)	45 (67.2)	<0.0001

Data are expressed as the median (interquartile) or number (percentage). α1MG, alpha1-microglobulin; ARB, angiotensin receptor blocker; ACEI, angiotensin-converting enzyme inhibitor; CCB, calcium channel blocker; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; GN, glomerulonephritis; MBP, mean blood pressure; UAE, urinary albumin excretion; uα1-MG, urinary α1-microglobulin; u5MedC, urinary 5-methyl-2'-deoxycytidine. *Chronic glomerulonephritis includes 93 cases (59.2%) of IgA nephropathy, 22 cases (14.0%) of minimal change nephrotic syndrome, 12 cases (7.6%) of membranous nephropathy, 12 cases (7.6%) of IgA vasculitis with nephritis, 7 cases (4.5%) of focal segmental glomerulosclerosis, 6 cases (3.8%) of non-IgA mesangial nephritis, 4 cases (2.6%) of membranoproliferative glomerulonephritis and 1 case (0.6%) of acute glomerulonephritis (persistent and chronic phase). **Others include 62 cases (62.0%) of unknown etiology without a renal biopsy; 20 cases (20.0%) of lupus nephritis; 10 cases (10.0%) of anti-neutrophil cytoplasmic antibody-associated vasculitis; 3 cases (3.0%) of polycystic kidney disease; 2 cases (2.0%) of Alport syndrome; and 1 case each (1.0%) of thin basement membrane disease, cholesterol crystal embolization, and vesicoureteral reflux.

of <0.05 was considered to be statistically significant. The SPSS version 20 software package (SPSS Inc., Chicago, IL, USA) and JMP version 11 program (SAS Institute Inc., Cary, NC, USA) were utilized to perform the statistical analyses.

3. Results

3.1. Urine 5MedC Levels and CKD Stages. The baseline profiles of the study subjects are summarized in accordance with the early to middle (stages 1 to 3) and later (stages 4 and 5) stages of CKD (Table 1). This study included 308 patients (male, $n = 164$; female, $n = 144$) with a median age of 56 (37-67) years. The background cause of CKD in more than half of the cases was chronic glomerulonephritis (51.0%). This distribution of patients with chronic glomerulonephritis was similar to that in other nephrology divisions reported in the Japan Renal Biopsy Registry [24]. Significant increases in the levels of albuminuria, urine α1MG, and MBP as well as significant decreases in hemoglobin were recognized, resembling those reported in other cohorts of CKD [6, 25, 26]. The median values of urine 5MedC were 59.7 and 88.3 μmol/gCr in the early to middle and later CKD stages, respectively (Figure 1). The concentrations of urine 5MedC were significantly increased in later stages of CKD, suggesting its association with disease progression (Figure 1).

3.2. A Multivariate Analysis to Determine a Low eGFR (Less than 30 mL/min/1.73 m^2) in Patients with CKD. Next, separate multiple logistic regression analyses to determine a low eGFR (<30 mL/min/1.73 m^2), which is equivalent to advanced CKD stages 4 and 5, were performed (Table 2). The urine 5MedC level alone was elucidated to be a significantly independent predictor of a low eGFR (model 1). After adjusting for gender and age as confounding parameters, the urine 5MedC level was still significant in model 2, which included albuminuria, and model 3, which further included uα1MG (Table 2). In the univariate analysis, there were no significant correlations between urine 5MedC and other parameters (Table S1). The urine 5MedC levels did not significantly differ when categorized according to the age, gender, cause of CKD, or complications (Table S2).

3.3. Urine 5MedC in Combination with Other Urine Proteins Significantly Predicts the Renal Survival. During the 36 months of follow-up, 46 patients exhibited a 30% decline in the eGFR ($n = 24$) or developed end-stage renal disease requiring renal replacement therapy ($n = 22$). There was a higher incidence of disease progression in patients with advanced CKD (stages 4 to 5) (33 of 67 patients) than in those with early to middle CKD (stages 1 to 3) (13 of 241 patients). The baseline levels of albuminuria (<300 mg/gCr or ≥300 mg/gCr) or urine α1MG (median

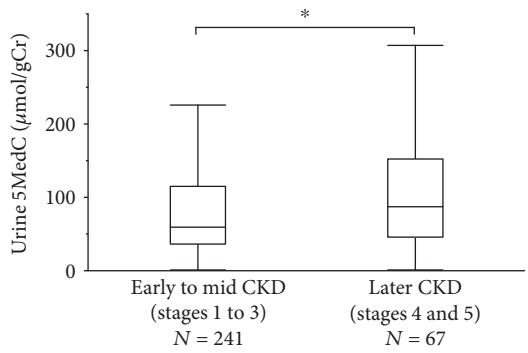

Figure 1: Urine 5MedC and CKD stages. Box and line plots showing the levels of urine 5MedC (μmol/gCr) according to the CKD stages (early to middle stages 1 to 3 or later stages 4 and 5) based on the estimated glomerular filtration rate. The boxes denote the medians and 25th and 75th percentiles. The lines mark the 5th and 95th percentiles. Wilcoxon's test. CKD: chronic kidney disease; 5MedC: 5-methyl-2′-deoxycytidine.

value, 5.7 mg/gCr) were able to predict the renal endpoint-free survival (Figure S1), suggesting that the CKD cohort in this study was consistent with the relative risk prediction model of CKD [6, 25, 26]. Several studies have investigated the combination of biomarkers to better predict the renal prognosis [19, 26]. We further performed survival analyses using the level of urine 5MedC (median value, 65.9 μmol/gCr) in combination with the level of urine albumin (<300 mg/gCr or ≥300 mg/gCr) or with that of urine α1MG (median value, 5.7 mg/gCr) in Kaplan-Meier survival curves (Figure 2). An increased urine 5MedC level in CKD patients did not significantly predict a worse renal outcome than a lower urine 5MedC level (Figure S1); however, a significant effect of an increased urine 5MedC level on predicting a poor renal survival when combined with macroalbuminuria (≥300 mg/gCr) or an increased urine α1MG level was observed (Figure 2).

3.4. Relationship between Urine 5MedC, a Marker of DNA Methylation, and Urine 8-OHdG, a Marker of Oxidized DNA. We carried out a further analysis of the 273 patients with available data for urine 8-OHdG, a marker of oxidized DNA due to oxidative stress. We recognized a significant univariate correlation between 5MedC and 8-OHdG in the urine of CKD patients, suggesting an association between DNA methylation and oxidized DNA and thus a linkage between epigenetic and genetic alterations in such patients (Figure 3).

4. Discussion

In the genomic DNA of mammals, methylation of the C-5 position of cytosine is a key mechanism of epigenetic control that influences the gene expression, stability of genome, and differentiation of cells [27]. Abnormal methylation of several genes, either hypermethylation or hypomethylation, has been involved in various diseases, including cancer [2, 28, 29], diabetes [30], obesity [31], Alzheimer's disease [32], and schizophrenia [5]. The level of 5-methylcytidine (5MeC) is determined by the balance between DNA methylation and DNA demethylation processes. DNA methylation is catalyzed by DNA methyltransferases, with S-adenosylmethionine functioning as a donor of methyl. DNA methylation may be removed enzymatically by certain mechanisms including BER [33], NER, and hydrolysis [34–36].

In this study, we measured the urine level of 5MedC, a marker of repair products of DNA methylation, in patients with CKD and investigated the relationships between the urine 5MedC level and CKD progression and outcomes. Herein, we provide the evidence that (1) the urine 5MedC level was significantly increased in the later stages of CKD (i.e., eGFR less than 30 mL/min/1.73 m^2) and was associated with a later CKD stage according to a multiple regression analysis even after adjusting for confounding parameters; furthermore, (2) while urine 5MedC alone did not significantly predict the renal outcome in CKD patients, a significant effect of urine 5MedC on predicting a poor renal outcome when combined with macroalbuminuria or an increased urine α1MG level was detected.

5MedC is a product of the BER and NER pathways of active DNA methylation. DNA repair products, including 5MedC, 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxycytosine, are released into the blood and subsequently appear in the urine [37, 38]. Several techniques have been developed for the determination of 5MedC in human urine samples, such as immunochemical detection [13], ion-pair liquid chromatography (LC) [16], LC with mass spectrometry (LC-MS) [14], LC with tandem mass spectrometry (LC-MS/MS) [12], and high-performance LC with tandem mass spectrometry (HPLC-MS/MS) [15, 39].

Itoh et al. reported that an ELISA with specific monoclonal antibodies was able to detect 5MedC as the major immunoreactive nucleoside in the urine of a healthy human and increased concentrations of urine 5MedC were observed in leukemic patients with active diseases [13]. The mean levels of urine 5MedC in healthy subjects were 0.90 ± 0.43 nmol/μmolCr in that analysis. The generation of 5MedC may be caused by the active excision repair of DNA in human cells. Heavily methylated DNA of leukemic cells may be the origin of increased 5MedC in the urine of leukemic patients. Zambonin et al. then applied a simple reversed-phase LC technique to determine the urine 5MedC levels normalized by urine creatinine excretion in healthy individuals and patients with leukemia [16]. Lee et al. further analyzed the urine levels of oxidized nucleosides using LC with electrospray mass spectrometry and found that the urine 5MedC levels did not significantly change, but those of 8-OHdG were significantly elevated in patients with Alzheimer's disease compared to healthy subjects [14]. The mean urine level of 5MedC was 0.262 ± 0.156 nmol/μmolCr in that study. Based on these previous findings, we initially excluded patients with cancer and Alzheimer's disease from the present study.

Hu et al. measured the level of urine 5MeC and 5MedC by LC-MS/MS with isotope dilution in healthy males and found that the concentration of urine 5MeC was significantly correlated with those of methylated purines and lesions of oxidized DNA, including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) [12]. The mean urine level of 5MedC was 7.04 ± 7.2 ng/mgCr in that report. The level of urine 5MedC,

Table 2: A multiple logistic regression analysis to determine low eGFR (later CKD stages, <30 mL/min/1.73 m^2) in different models.

	Odds ratio	95% CI	*P* value
Model 1			
u5MedC ≥ median (μmol/gCr)	2.30	1.29 - 4.21	0.005
Model 2			
u5MedC ≥ median (μmol/gCr)	2.16	1.18 – 4.04	0.012
UAE ≥ 300 (mg/gCr)	4.31	2.36 – 8.08	<0.0001
Model 3			
u5MedC ≥ median (μmol/gCr)	2.36	1.24 - 4.60	0.008
UAE ≥ 300 (mg/gCr)	1.39	0.67 - 2.90	0.381
uα1MG ≥ median (mg/gCr)	13.56	5.32 – 40.1	<0.0001

Adjusted for age and gender. The median values of u5MedC and uα1MG are 65.9 (μmol/gCr) and 5.7 (mg/gCr), respectively. CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; UAE, urinary albumin excretion; uα1-MG, urinary α1-microglobulin; u5MedC, urinary 5-methyl-2'-deoxycytidine; CI, confidence interval.

however, did not correlate with any methylated or oxidized lesions in healthy male subjects in that study. Pan et al. investigated the levels of urine 5MedC and 5-hydroxymethyl-2′-deoxycytidine (5hMedC) by HPLC-MS/MS in subfertile men and showed their associations with phthalate metabolites (environmental chemicals) and semen parameters (healthy outcomes), suggesting that these are promising biomarkers for use in epidemiological studies [15, 39]. In addition to urine samples of humans, other researchers have attempted to determine the 5MedC level in DNA obtained from human peripheral blood [40] or human lung cancer tissue [41] by LC-MS/MS as well as in DNA obtained from cultured Hela cells by HPLC-ultraviolet detection [42] and from newborn cord blood samples by HPLC-MS/MS [43].

Recent reports have identified roles of environmental [6], genetic [44, 45], and epigenetic factors [46, 47] in the progression of CKD. Epigenetic risk factors for CKD have only recently been investigated [10], and the DNA methylation profile in the blood might be associated with a rapid decline in the renal function [48]. In the present study, the group exhibiting both higher levels of urine 5MedC and albuminuria had a worse renal survival than the group exhibiting lower levels of both (Figure 2). Whether albuminuria induces epigenetic changes, including DNA methylation, and thus an increase in urine 5MedC in resident kidney cells is largely unknown. The expression of Krüppel-like factor 4, which can reprogram somatic cells into induced pluripotent stem cells, reduced DNA methylation at the nephrin promoter, which may lead to protection against albuminuria [49]. The hypomethylation of *aldo-keto reductase family 1 member B1* and *tissue inhibitor of metalloproteinase 2* genes in association with albuminuria has been reported in subjects with early stages of diabetic nephropathy [50], although we did not recognize a significant correlation between urine 5MedC and albuminuria levels in the univariate analysis in our cohort (Table S1).

We found in the present study that urine 5MedC levels were significantly increased in the later stages of CKD (stages 4 and 5, i.e., eGFR less than 30 mL/min/1.73 m^2) (Figure 1), when uremic toxins may be detected in both the urine and serum of such patients. In recent reports, uremia was shown to induce alterations in DNA methylation in differentiating monocytes in patients with CKD [51]. The expression of the antiaging and renoprotective gene *klotho* is known to be suppressed under conditions of uremia [18]. The protein-bound uremic toxins can increase the DNA methyltransferase and DNA methylation, thereby leading to the suppression of the *klotho* expression in the uremic milieu [52]. Therefore, certain uremic toxins might alter the global DNA methylation and the expression of urine 5MedC in CKD patients. In rodent models, hypermethylation of certain genes is involved in the activation of fibroblasts and fibrogenesis in the kidney, which may be one of the molecular mechanisms associated with the progression of CKD [53].

Epigenetic patterns can change over one's lifetime, suggesting that epigenetic changes may constitute an important factor of the aging process [54]. Since CKD might be an aging-related disorder, we investigated the urine 5MedC level in different age categories in our CKD cohort. However, the CKD patients ≥ 75 years of age did not exhibit a significantly different level of urine 5MedC than those <75 years of age in our study (Table S2). We recognized the correlation between urine 5MedC, a marker of global DNA methylation, and urine 8-OHdG, a marker of oxidized DNA by oxidative stress (Figure 3), suggesting a link between DNA oxidation and DNA methylation. There might therefore be a connection between genetic and epigenetic alterations, possibly via oxidative stress in such patients. Several reports have investigated the relationship between oxidized DNA and DNA methylation [55–58], including the simultaneous examination of 8-OHdG and 5MedC in DNA samples [55]. 8-OHdG may induce hypomethylation of DNA by inhibiting DNA methylation at nearby cytosine bases [58]. Significantly negative correlations were reported between 8-OHdG and levels of global methylation in DNA extracted from leukocytes in workers exposed to nanomaterials of metal oxide [56] and between plasma 8-OHdG and global methylation levels in leukocyte DNA in subjects with biliary atresia [57]. Further investigations are thus required in order to clarify the association between oxidized DNA and DNA methylation.

This study has several limitations and strengths that must be kept in mind when understanding the data. First, urine 5MedC did not exhibit methylation of specific genes involved

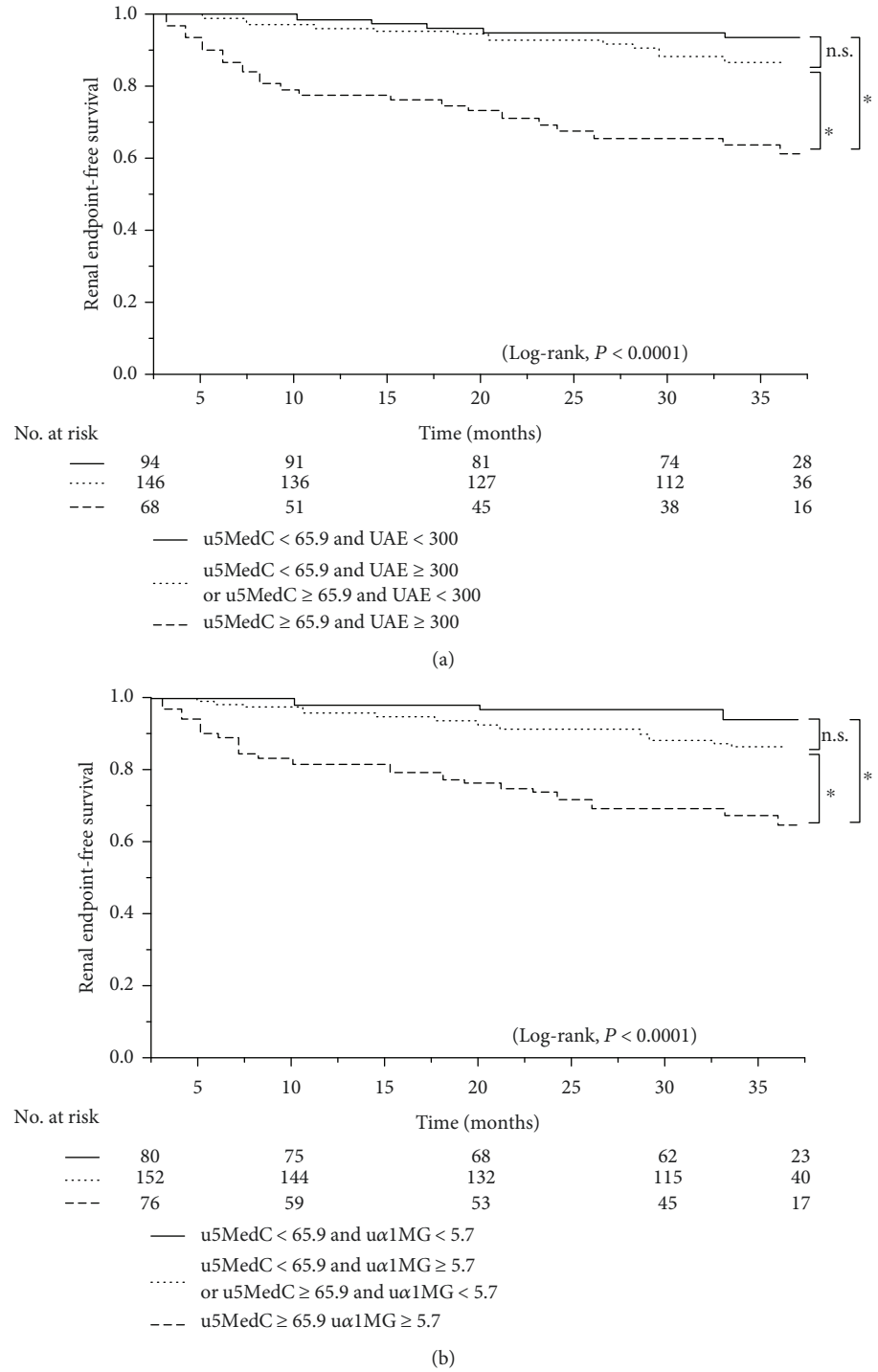

Figure 2: Urine 5MedC and CKD outcome. Kaplan-Meier curves showing the renal endpoint-free survival categorized by urine 5MedC (μmol/gCr) and its combination with albuminuria (mg/gCr) (a) or urine α1MG (mg/gCr) (b). The combination of urine 5MedC with albuminuria (a) or urine α1MG (b) clearly separated the three-year renal endpoint-free survival of CKD patients. (a) u5MedC < 65.9 and UAE < 300, $n = 94$ (30.5%); u5MedC < 65.9 and UAE ≥ 300 or u5MedC ≥ 65.9 and UAE < 300, $n = 146$ (47.4%); and u5MedC ≥ 65.9 and UAE ≥ 300, $n = 68$ (22.1%). (b) u5MedC < 65.9 and uα1MG < 5.7, $n = 80$ (26.0%); u5MedC < 65.9 and uα1MG ≥ 5.7 or u5MedC ≥ 65.9 and uα1MG < 5.7, $n = 152$ (49.4%); u5MedC ≥ 65.9 and uα1MG ≥ 5.7, $n = 76$ (24.7%). $*$ indicates $P < 0.0001$, n.s. denotes not significant. Log-rank test. UAE: urinary albumin excretion; uα1MG: urinary alpha1-microglobulin; u5MedC: urinary 5-methyl-2′-deoxycytidine.

in CKD, such as *polycystic kidney disease 1* [59] but exhibited global DNA methylation. Second, while three major enzymes are necessary for de novo DNA methylation (DNMT3A and DNMT3B) or maintenance methylation (DNMT1) in mammalian cells [3], we did not examine the levels of these enzymes in the present study. Third, we did not have

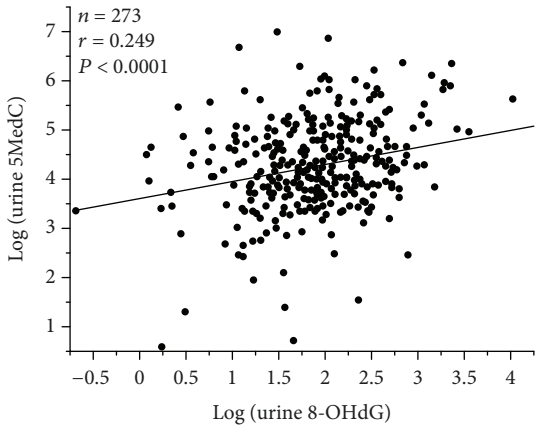

Figure 3: Relationship between levels of urine 5MedC and urine 8-OHdG. The level of urine 5MedC (μmol/gCr) significantly correlates with the level of urine 8-OHdG (ng/mgCr) in patients with CKD ($n = 273$). t-test. 5MedC: 5-methyl-2′-deoxycytidine; 8-OHdG: 8-hydroxy-2′-deoxyguanosine.

sufficient data on subjects with diabetic nephropathy, which is the most frequent cause of ESRD in several countries. However, including diabetic subjects in the CKD cohort may have influenced the 5MedC levels, as alterations in the DNA methylation of the gene network occur under conditions of diabetes mellitus [60] and in glomerular podocytes under conditions of diabetic nephropathy [61, 62]. Fourth, the serum and kidney tissue levels of 5MedC were not investigated in this study, as we did not obtain these samples in our setting. Renal compartment-specific genetic and epigenetic analyses would be able to identify further novel mechanisms involved in the progression of CKD [63]. Fifth, we were unable to evaluate cardiovascular events in CKD patients because we expected a low number of such events in our setting, although epigenetic dysregulation of CKD-associated cardiovascular disease might be relevant [64]. In addition, we were unable to confirm the level of 5MedC in our urine samples using other methodologies, such as LC-MS/MS [12]. Other caveats include the lack of data on lifestyle risks, such as smoking and toxin exposure, as potential confounders.

5. Conclusions

We examined the levels of urine 5MedC, a marker of DNA methylation and an epigenetic marker, in patients with CKD. These values significantly increased in the later CKD stages and were related to a reduced eGFR. The urine 5MedC level in combination with albuminuria or the α1MG level significantly predicted the renal survival in CKD patients, suggesting that it can serve as a novel biomarker for predicting the renal outcome in CKD, which is a significant issue given the currently increasing number of CKD patients all over the world. Our previous studies and others suggested urine trefoil factors to be biomarkers for progression of CKD [19, 65, 66]; however, recent studies demonstrated these small peptides as biomarkers for acute kidney injury [67] and drug-induced kidney injury [68]. We thus believe urine 5MedC as a promising and novel biomarker for CKD based on the current study.

Further studies to clarify the kidney disease-specific changes in the level of 5MedC utilizing a larger CKD cohort and exploring the renal compartment-specific epigenetic analyses will be necessary. Clarifying whether intervention and treatment of CKD patients with agents such as cholesterol-lowering medications [69] can alter the level of urine 5MedC is of great importance.

Data Availability

No data is used to support this study.

Conflicts of Interest

Jun Wada takes honoraria as a speaker from Daiichi Sankyo, MSD, Tanabe Mitsubishi, and Taisho Toyama and receives support from a grant from Baxter, Dainippon Sumitomo, Ono, and Teijin Pharma. Haruhito A. Uchida belongs to the Department of Chronic Kidney Disease and Cardiovascular Disease which is supported by Chugai Pharmaceutical, MSD, Boehringer Ingelheim, and Kawanishi Holdings. The other authors declare that they have no competing interests.

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Supplementary Materials

Supplementary Table S1: univariate correlation between urinary 5MedC and other clinical parameters. Supplementary Table S2: urinary 5MedC levels according to age, gender, cause of CKD, and complication of diabetes. Supplementary Figure S1: the renal survival categorized by albuminuria, urine α1MG, and urine 5MedC alone. *(Supplementary Materials)*

References

[1] A. P. Feinberg and M. D. Fallin, "Epigenetics at the crossroads of genes and the environment," *JAMA*, vol. 314, no. 11, pp. 1129-1130, 2015.

[2] P. A. Jones and S. B. Baylin, "The epigenomics of cancer," *Cell*, vol. 128, no. 4, pp. 683–692, 2007.

[3] T. Chen and E. Li, "Structure and function of eukaryotic DNA methyltransferases," *Current Topics in Developmental Biology*, vol. 60, pp. 55–89, 2004.

[4] K. D. Robertson, "DNA methylation and human disease," *Nature Reviews Genetics*, vol. 6, no. 8, pp. 597–610, 2005.

[5] C. Montano, M. A. Taub, A. Jaffe et al., "Association of DNA methylation differences with schizophrenia in an epigenome-wide association study," *JAMA Psychiatry*, vol. 73, no. 5, pp. 506–514, 2016.

[6] A. S. Levey and J. Coresh, "Chronic kidney disease," *The Lancet*, vol. 379, no. 9811, pp. 165–180, 2012.

[7] K. F. National, "K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification," *American Journal of Kidney Diseases: The Official Journal of the National Kidney Foundation*, vol. 39, no. 2, Supplement 1, p. S1, 2002.

[8] B. C. Astor, K. Matsushita, R. T. Gansevoort et al., "Lower estimated glomerular filtration rate and higher albuminuria are associated with mortality and end-stage renal disease. A collaborative meta-analysis of kidney disease population cohorts," *Kidney International*, vol. 79, no. 12, pp. 1331–1340, 2011.

[9] R. G. Fassett, S. K. Venuthurupalli, G. C. Gobe, J. S. Coombes, M. A. Cooper, and W. E. Hoy, "Biomarkers in chronic kidney disease: a review," *Kidney International*, vol. 80, no. 8, pp. 806–821, 2011.

[10] L. J. Smyth, G. J. McKay, A. P. Maxwell, and A. J. McKnight, "DNA hypermethylation and DNA hypomethylation is present at different loci in chronic kidney disease," *Epigenetics*, vol. 9, no. 3, pp. 366–376, 2014.

[11] S. Kato, B. Lindholm, P. Stenvinkel et al., "DNA hypermethylation and inflammatory markers in incident Japanese dialysis patients," *Nephron Extra*, vol. 2, no. 1, pp. 159–168, 2012.

[12] C. W. Hu, H. H. Liu, Y. J. Li, and M. R. Chao, "Direct analysis of 5-methylcytosine and 5-methyl-2′-deoxycytidine in human urine by isotope dilution LC-MS/MS: correlations with N-methylated purines and oxidized DNA lesions," *Chemical Research in Toxicology*, vol. 25, no. 2, pp. 462–470, 2012.

[13] K. Itoh, S. Aida, S. Ishiwata, T. Yamaguchi, N. Ishida, and M. Mizugaki, "Immunochemical detection of urinary 5-methyl-2′-deoxycytidine as a potential biologic marker for leukemia," *Clinica Chimica Acta*, vol. 234, no. 1-2, pp. 37–45, 1995.

[14] S. H. Lee, I. Kim, and B. C. Chung, "Increased urinary level of oxidized nucleosides in patients with mild-to-moderate Alzheimer's disease," *Clinical Biochemistry*, vol. 40, no. 13-14, pp. 936–938, 2007.

[15] Y. Pan, J. Jing, L. W. Yeung et al., "Associations of urinary 5-methyl-2′-deoxycytidine and 5-hydroxymethyl-2′-deoxycytidine with phthalate exposure and semen quality in 562 Chinese adult men," *Environment International*, vol. 94, pp. 583–590, 2016.

[16] C. G. Zambonin, A. Aresta, F. Palmisano, G. Specchia, and V. Liso, "Liquid chromatographic determination of urinary 5-methyl-2′-deoxycytidine and pseudouridine as potential biological markers for leukaemia," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 21, no. 5, pp. 1045–1051, 1999.

[17] S. Matsuo, E. Imai, M. Horio et al., "Revised equations for estimated GFR from serum creatinine in Japan," *American Journal of Kidney Diseases*, vol. 53, no. 6, pp. 982–992, 2009.

[18] M. Kitagawa, H. Sugiyama, H. Morinaga et al., "A decreased level of serum soluble klotho is an independent biomarker associated with arterial stiffness in patients with chronic kidney disease," *PLoS One*, vol. 8, no. 2, article e56695, 2013.

[19] T. Yamanari, H. Sugiyama, K. Tanaka et al., "Urine trefoil factors as prognostic biomarkers in chronic kidney disease," *BioMed Research International*, vol. 2018, Article ID 3024698, 11 pages, 2018.

[20] N. Fukuoka, H. Sugiyama, T. Inoue et al., "Increased susceptibility to oxidant-mediated tissue injury and peritoneal fibrosis in acatalasemic mice," *American Journal of Nephrology*, vol. 28, no. 4, pp. 661–668, 2008.

[21] H. Morinaga, H. Sugiyama, T. Inoue et al., "Effluent free radicals are associated with residual renal function and predict technique failure in peritoneal dialysis patients," *Peritoneal Dialysis International*, vol. 32, no. 4, pp. 453–461, 2012.

[22] J. Coresh, T. C. Turin, K. Matsushita et al., "Decline in estimated glomerular filtration rate and subsequent risk of end-stage renal disease and mortality," *JAMA*, vol. 311, no. 24, pp. 2518–2531, 2014.

[23] E. Kanda, T. Usui, N. Kashihara, C. Iseki, K. Iseki, and M. Nangaku, "Importance of glomerular filtration rate change as surrogate endpoint for the future incidence of end-stage renal disease in general Japanese population: community-based cohort study," *Clinical and Experimental Nephrology*, vol. 22, no. 2, pp. 318–327, 2018.

[24] Committee for Standardization of Renal Pathological Diagnosis and Committee for Kidney Disease Registry, Japanese Society of Nephrology, Japan, H. Sugiyama, H. Yokoyama et al., "Japan Renal Biopsy Registry and Japan Kidney Disease Registry: committee report for 2009 and 2010," *Clinical and Experimental Nephrology*, vol. 17, no. 2, pp. 155–173, 2013.

[25] A. S. Levey, P. E. de Jong, J. Coresh et al., "The definition, classification, and prognosis of chronic kidney disease: a KDIGO controversies conference report," *Kidney International*, vol. 80, no. 1, pp. 17–28, 2011.

[26] Y. Otaki, T. Watanabe, T. Shishido et al., "The impact of renal tubular damage, as assessed by urinary β_2-microglobulin-creatinine ratio, on cardiac prognosis in patients with chronic heart failure," *Circulation: Heart Failure*, vol. 6, no. 4, pp. 662–668, 2013.

[27] M. Gehring, W. Reik, and S. Henikoff, "DNA demethylation by DNA repair," *Trends in Genetics*, vol. 25, no. 2, pp. 82–90, 2009.

[28] M. Hatziapostolou and D. Iliopoulos, "Epigenetic aberrations during oncogenesis," *Cellular and Molecular Life Sciences*, vol. 68, no. 10, pp. 1681–1702, 2011.

[29] B. Thienpont, J. Steinbacher, H. Zhao et al., "Tumour hypoxia causes DNA hypermethylation by reducing TET activity," *Nature*, vol. 537, no. 7618, pp. 63–68, 2016.

[30] L. Sommese, A. Zullo, F. P. Mancini, R. Fabbricini, A. Soricelli, and C. Napoli, "Clinical relevance of epigenetics in the onset and management of type 2 diabetes mellitus," *Epigenetics*, vol. 12, no. 6, pp. 401–415, 2017.

[31] M. M. Mendelson, R. E. Marioni, R. Joehanes et al., "Association of body mass index with DNA methylation and gene expression in blood cells and relations to cardiometabolic disease: a Mendelian randomization approach," *PLoS Medicine*, vol. 14, no. 1, article e1002215, 2017.

[32] C. T. Watson, P. Roussos, P. Garg et al., "Genome-wide DNA methylation profiling in the superior temporal gyrus reveals epigenetic signatures associated with Alzheimer's disease," *Genome Medicine*, vol. 8, no. 1, p. 5, 2016.

[33] M. Vairapandi and N. J. Duker, "Enzymic removal of 5-methylcytosine from DNA by a human DNA-glycosylase," *Nucleic Acids Research*, vol. 21, no. 23, pp. 5323–5327, 1993.

[34] G. Barreto, A. Schäfer, J. Marhold et al., "Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation," *Nature*, vol. 445, no. 7128, pp. 671–675, 2007.

[35] S. K. Bhattacharya, S. Ramchandani, N. Cervoni, and M. Szyf, "A mammalian protein with specific demethylase

activity for mCpG DNA," *Nature*, vol. 397, no. 6720, pp. 579–583, 1999.

[36] J. K. Zhu, "Active DNA demethylation mediated by DNA glycosylases," *Annual Review of Genetics*, vol. 43, no. 1, pp. 143–166, 2009.

[37] S. Kriaucionis and N. Heintz, "The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain," *Science*, vol. 324, no. 5929, pp. 929-930, 2009.

[38] M. Tahiliani, K. P. Koh, Y. Shen et al., "Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1," *Science*, vol. 324, no. 5929, pp. 930–935, 2009.

[39] R. Yin, J. Mo, M. Lu, and H. Wang, "Detection of human urinary 5-hydroxymethylcytosine by stable isotope dilution HPLC-MS/MS analysis," *Analytical Chemistry*, vol. 87, no. 3, pp. 1846–1852, 2015.

[40] X. Li and A. A. Franke, "High-throughput and cost-effective global DNA methylation assay by liquid chromatography-mass spectrometry," *Analytica Chimica Acta*, vol. 703, no. 1, pp. 58–63, 2011.

[41] C.-W. Hu, H. Lee, J.-L. Chen, Y.-J. Li, and M.-R. Chao, "Optimization of global DNA methylation measurement by LC-MS/MS and its application in lung cancer patients," *Analytical and Bioanalytical Chemistry*, vol. 405, no. 27, pp. 8859–8869, 2013.

[42] J. Sandhu, B. Kaur, C. Armstrong et al., "Determination of 5-methyl-2′-deoxycytidine in genomic DNA using high performance liquid chromatography-ultraviolet detection," *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, vol. 877, no. 20-21, pp. 1957–1961, 2009.

[43] P. Intarasunanont, P. Navasumrit, S. Waraprasit et al., "Effects of arsenic exposure on DNA methylation in cord blood samples from newborn babies and in a human lymphoblast cell line," *Environmental Health*, vol. 11, no. 1, 2012.

[44] J. J. Grantham, V. E. Torres, A. B. Chapman et al., "Volume progression in polycystic kidney disease," *New England Journal of Medicine*, vol. 354, no. 20, pp. 2122–2130, 2006.

[45] A. Parsa, W. H. Kao, D. Xie et al., "APOL1 risk variants, race, and progression of chronic kidney disease," *The New England Journal of Medicine*, vol. 369, no. 23, pp. 2183–2196, 2013.

[46] P. Stenvinkel, M. Karimi, S. Johansson et al., "Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease?," *Journal of Internal Medicine*, vol. 261, no. 5, pp. 488–499, 2007.

[47] K. Susztak, "Understanding the epigenetic syntax for the genetic alphabet in the kidney," *Journal of the American Society of Nephrology*, vol. 25, no. 1, pp. 10–17, 2014.

[48] M. R. Wing, J. M. Devaney, M. M. Joffe et al., "DNA methylation profile associated with rapid decline in kidney function: findings from the CRIC study," *Nephrology, Dialysis, Transplantation*, vol. 29, no. 4, pp. 864–872, 2014.

[49] K. Hayashi, H. Sasamura, M. Nakamura et al., "KLF4-dependent epigenetic remodeling modulates podocyte phenotypes and attenuates proteinuria," *The Journal of Clinical Investigation*, vol. 124, no. 6, pp. 2523–2537, 2014.

[50] O. Aldemir, F. Turgut, and C. Gokce, "The association between methylation levels of targeted genes and albuminuria in patients with early diabetic kidney disease," *Renal Failure*, vol. 39, no. 1, pp. 597–601, 2017.

[51] A. M. Zawada, J. S. Schneider, A. I. Michel et al., "DNA methylation profiling reveals differences in the 3 human monocyte subsets and identifies uremia to induce DNA methylation changes during differentiation," *Epigenetics*, vol. 11, no. 4, pp. 259–272, 2016.

[52] C. Y. Sun, S. C. Chang, and M. S. Wu, "Suppression of klotho expression by protein-bound uremic toxins is associated with increased DNA methyltransferase expression and DNA hypermethylation," *Kidney International*, vol. 81, no. 7, pp. 640–650, 2012.

[53] W. Bechtel, S. McGoohan, E. M. Zeisberg et al., "Methylation determines fibroblast activation and fibrogenesis in the kidney," *Nature Medicine*, vol. 16, no. 5, pp. 544–550, 2010.

[54] M. J. Jones, S. J. Goodman, and M. S. Kobor, "DNA methylation and healthy human aging," *Aging Cell*, vol. 14, no. 6, pp. 924–932, 2015.

[55] J. Hu, W. Zhang, H. Ma, Y. Cai, G. Sheng, and J. Fu, "Simultaneous determination of 8-hydroxy-2′-deoxyguanosine and 5-methyl-2′-deoxycytidine in DNA sample by high performance liquid chromatography/positive electrospray ionization tandem mass spectrometry," *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, vol. 878, no. 28, pp. 2765–2769, 2010.

[56] S. H. Liou, W. T. Wu, H. Y. Liao et al., "Global DNA methylation and oxidative stress biomarkers in workers exposed to metal oxide nanoparticles," *Journal of Hazardous Materials*, vol. 331, pp. 329–335, 2017.

[57] W. Udomsinprasert, N. Kitkumthorn, A. Mutirangura, V. Chongsrisawat, Y. Poovorawan, and S. Honsawek, "Global methylation, oxidative stress, and relative telomere length in biliary atresia patients," *Scientific Reports*, vol. 6, no. 1, article 26969, 2016.

[58] Q. Wu and X. Ni, "ROS-mediated DNA methylation pattern alterations in carcinogenesis," *Current Drug Targets*, vol. 16, no. 1, pp. 13–19, 2015.

[59] Y. M. Woo, J. B. Bae, Y. H. Oh et al., "Genome-wide methylation profiling of ADPKD identified epigenetically regulated genes associated with renal cyst development," *Human Genetics*, vol. 133, no. 3, pp. 281–297, 2014.

[60] J. Kang, C. N. Lee, H. Y. Li, K. H. Hsu, and S. Y. Lin, "Genome-wide DNA methylation variation in maternal and cord blood of gestational diabetes population," *Diabetes Research and Clinical Practice*, vol. 132, pp. 127–136, 2017.

[61] K. Hasegawa, S. Wakino, P. Simic et al., "Renal tubular Sirt1 attenuates diabetic albuminuria by epigenetically suppressing claudin-1 overexpression in podocytes," *Nature Medicine*, vol. 19, no. 11, pp. 1496–1504, 2013.

[62] T. Marumo, S. Yagi, W. Kawarazaki et al., "Diabetes induces aberrant DNA methylation in the proximal tubules of the kidney," *Journal of the American Society of Nephrology*, vol. 26, no. 10, pp. 2388–2397, 2015.

[63] C. Qiu, S. Huang, J. Park et al., "Renal compartment-specific genetic variation analyses identify new pathways in chronic kidney disease," *Nature Medicine*, vol. 24, no. 11, pp. 1721–1731, 2018.

[64] A. M. Zawada, K. S. Rogacev, and G. H. Heine, "Clinical relevance of epigenetic dysregulation in chronic kidney disease-associated cardiovascular disease," *Nephrology Dialysis Transplantation*, vol. 28, no. 7, pp. 1663–1671, 2013.

[65] D. Lebherz-Eichinger, B. Tudor, H. J. Ankersmit et al., "Increased trefoil factor 2 levels in patients with chronic kidney disease," *PLoS One*, vol. 12, no. 3, article e0174551, 2017.

[66] K. Tanaka, H. Sugiyama, T. Yamanari et al., "Renal expression of trefoil factor 3 mRNA in association with tubulointerstitial fibrosis in IgA nephropathy," *Nephrology (Carlton)*, vol. 23, no. 9, pp. 855–862, 2018.

[67] T. C. Spada, J. M. R. D. Silva, L. S. Francisco et al., "High intensity resistance training causes muscle damage and increases biomarkers of acute kidney injury in healthy individuals," *PLoS One*, vol. 13, no. 11, article e0205791, 2018.

[68] B. R. Griffin, S. Faubel, and C. L. Edelstein, "Biomarkers of drug-induced kidney toxicity," *Therapeutic Drug Monitoring*, vol. 41, no. 2, pp. 213–226, 2019.

[69] A. Zinellu, S. Sotgia, E. Sotgiu et al., "Cholesterol lowering treatment restores blood global DNA methylation in chronic kidney disease (CKD) patients," *Nutrition, Metabolism, and Cardiovascular Diseases*, vol. 27, no. 9, pp. 822–829, 2017.

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Review Article

The Prognostic Role of Klotho in Patients with Chronic Kidney Disease: A Systematic Review and Meta-analysis

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Objective. The prognostic role of Klotho in patients with chronic kidney disease is still controversial. Therefore, we performed this meta-analysis to assess the relationship between the low sKlotho level and the risk of adverse kidney outcomes. *Materials and Methods*. We systematically searched medical databases, such as PubMed, Embase, and the Cochrane Library, for eligible publications regarding the relationship between the low sKlotho level and risk of adverse kidney outcomes. The quality of included studies was assessed by using the Newcastle–Ottawa Scale. Combined hazard ratios (HRs) and its 95% confidence intervals (CIs) were calculated using a random- or fixed-effect model. Subgroup analysis was conducted with stratification by age, estimated glomerular filtration rate (eGFR), follow-up interval, region, and study quality. All data was analyzed by RevMan 5.3 analysis software. *Results*. Eight cohort studies with 3586 participants from 3818 studies were included in our final analysis. Levels of sKlotho were significantly correlated with the eGFR, with a summary correlation coefficient r and 95% CI of 0.469 (0.226, 0.658). Additionally, low sKlotho levels were strongly associated with increased adverse kidney outcomes, and the pooled HR and its 95% CI were 1.64 (1.19, 2.26; $P = 0.002$), despite publication bias and statistical heterogeneity ($I^2 = 52\%$, $P = 0.07$). Furthermore, this positive correlation was still observed in all of the subgroup analyses. However, heterogeneity was present in subgroup analyses stratified by the eGFR and follow-up interval. *Conclusion*. Levels of sKlotho are positively correlated with the eGFR, and low sKlotho levels are significantly associated with an increased risk of poor kidney outcomes. Therefore, sKlotho could be used as a novel biomarker for early diagnosis and prognostic assessment for patients with chronic kidney disease. Studies with a larger sample size and longer follow-up period are warranted to validate our results.

1. Introduction

Kuro-o et al. identified α-Klotho in 1997 as a novel antiaging gene [1], encoding two Klotho proteins. One of these proteins is the membrane-bond form (mKlotho), a single-pass membrane protein, which is expressed on the cell surface. The short extracellular domain of mKlotho can be cleaved by proteases and released into the blood. This shed extracellular domain is defined as soluble or secreted Klotho (sKlotho) [2]. mKlotho and sKlotho possess distinct biological functions [3]. mKlotho forms a complex with the fibroblast growth factor receptor (FGFR) and serves as the coreceptor for the fibroblast growth factor 23 (FGF23) to maintain mineral homeostasis [3, 4]. sKlotho can be detected in the circulation by ELISA assay [5] and is believed to be a main active form. sKlotho exerts pleiotropic beneficial effects by acting as a circulating hormone and protects cells against oxidative stress, hypoxia, and inflammation and inhibits cell apoptosis and organ fibrosis [6]. The Klotho gene is located in multiple organs including the kidney, brain, parathyroid, testis, and pituitary gland [1, 7, 8]. Among these organs, the kidney has the highest Klotho levels, indicating that the

kidney is the major organ which generates Klotho [9]. Therefore, unsurprisingly, Klotho levels decrease if the organ of origin is diseased [10, 11].

Chronic kidney disease (CKD) is increasingly considered as a major public health issue worldwide with high mortality and morbidity rates [12]. Currently, there is no effective therapy available for treating CKD. Therefore, early detection or prognosis is important for the prevention and treatment of CKD. However, there is no standard biomarker for early diagnosis and the monitoring of disease exacerbation in the course of CKD. Emerging evidence from patients with CKD has shown that sKlotho levels are decreased in the early stages of CKD, and they further decline as CKD progresses [11, 13–15]. Moreover, reduced sKlotho levels are associated with an elevated risk of deterioration in renal function or renal replacement treatment (RRT) [13, 16]. Therefore, sKlotho is proposed as a biomarker for the early diagnosis and progression of CKD. A correlation between sKlotho levels and kidney function has been recently systematically reviewed by Wang et al. [17]. These authors showed a positive association between sKlotho levels and the estimated glomerular filtration rate (eGFR), and they evaluated the possibility of sKlotho as an early biomarker for CKD early diagnosis. However, the role of sKlotho in predicting adverse outcomes in the kidney remains controversial [18]. Therefore, we performed a meta-analysis to assess the prognostic role of sKlotho by investigating the association between sKlotho levels and progression of CKD.

2. Materials and Methods

2.1. Search Strategy. A systematic literature search of PubMed, Embase, and the Cochrane Library was performed by two authors. The search was restricted to articles written in English. The terms that were used for the search were as follows: ([Klotho or alpha-Klotho or α-Klotho or αKL] and [chronic kidney disease or CKD or chronic kidney insufficiency or chronic kidney failure or chronic nephropathy or chronic kidney dysfunction] or [biomarker or marker or prognosis or outcome or progression or decline or deterioration]). Moreover, the reference lists of included studies were also retrieved manually for additional relevant studies. The updated date was January 15, 2019.

2.2. Inclusion Criteria and Exclusion Criteria. We included studies on the basis of the inclusion and exclusion criteria. Inclusion criteria were as follows: (1) a cohort or cohort and observational study, (2) a study that investigated the association between sKlotho levels and adverse kidney outcomes in patients with CKD, and (3) the patient's age was ≥18 years. Exclusion criteria were as follows: (1) an observational study, (2) a study that investigated the relationship between renal Klotho or urinary sKlotho levels and kidney function or other parameters, (3) a study with incomplete data, (4) patients with kidney transplantation or dialysis, (5) an animal experimental study (*in vivo* or *in vitro*), and (6) case reports, posters, editorials, and reviews.

2.3. Study Selection. Two authors independently screened the abstracts and titles of the relevant studies and eliminated studies that were not applicable according to the prestated inclusion criteria. However, reviews that possibly contained relevant information were initially included. The same two authors independently assessed the eligibility of the remaining full-text articles. Disagreements regarding study selection were resolved by discussion with an arbitrator.

2.4. Data Extraction. Two investigators extracted data independently from the included literature using a standardized data extraction form. The extracted content for each study included the first author's name, year of publication, study design, sample size, age, research region, assay use, correlation coefficient (Pearson or Spearman), hazard ratio (HR), odds ratio (OR), and 95% confidence interval (CI). The estimated HRs were acquired from the Kaplan–Meier curves as previously described [19] if the HRs were not obtained directly in the studies. Discrepancies in data extraction were addressed by consulting a third arbitrator. The most complete data were used if more than one publication of one study existed. If the data were not obtained or not complete, the first or corresponding author was contacted by e-mail.

2.5. Quality Assessment. The quality of included studies was independently assessed by three authors using the Newcastle–Ottawa Scale (NOS) [20]. Studies with ≥8 awarded stars were considered as high-quality studies. Disagreements among authors were resolved by discussing with an independent third party. The quality items assessed were eight items including patient selection, comparability, and outcome.

2.6. Meta-analysis. Pearson correlation coefficients were converted into Spearman correlation coefficients, and the latter were used for estimating the associations between sKlotho levels and the eGFR [17, 19]. Correlation coefficients underwent Fisher's Z transformation to generate a Z value, and then we calculated the standard error of Z. Meta-analysis was used to obtain the summary Fisher's Z value, and this was then transformed back by inverse Fisher's transformation to obtain the summary effect size (r) and 95% CIs. The pooled HRs and corresponding 95% CIs were used to evaluate the effect of sKlotho levels on adverse kidney outcomes. Meta-analysis was performed by Review Manager 5.3 analysis software (Cochrane Collaboration, Copenhagen, Denmark). Heterogeneity across included studies was analyzed using I^2 statistics. The fixed-effect model was used when the I^2 value was <50%. The random-effect model was applied when the I^2 value was >50%. Subgroup analysis was performed to examine the source of heterogeneity. The stability of the results was evaluated by sensitivity analysis via switching between the fixed-effect and the random-effect model. Potential publication bias was assessed by using the funnel plot.

3. Results

3.1. Study Selection. A total of 3818 relevant publications were extracted by searching databases, including PubMed, Embase, and the Cochrane Library. Of these studies, 615

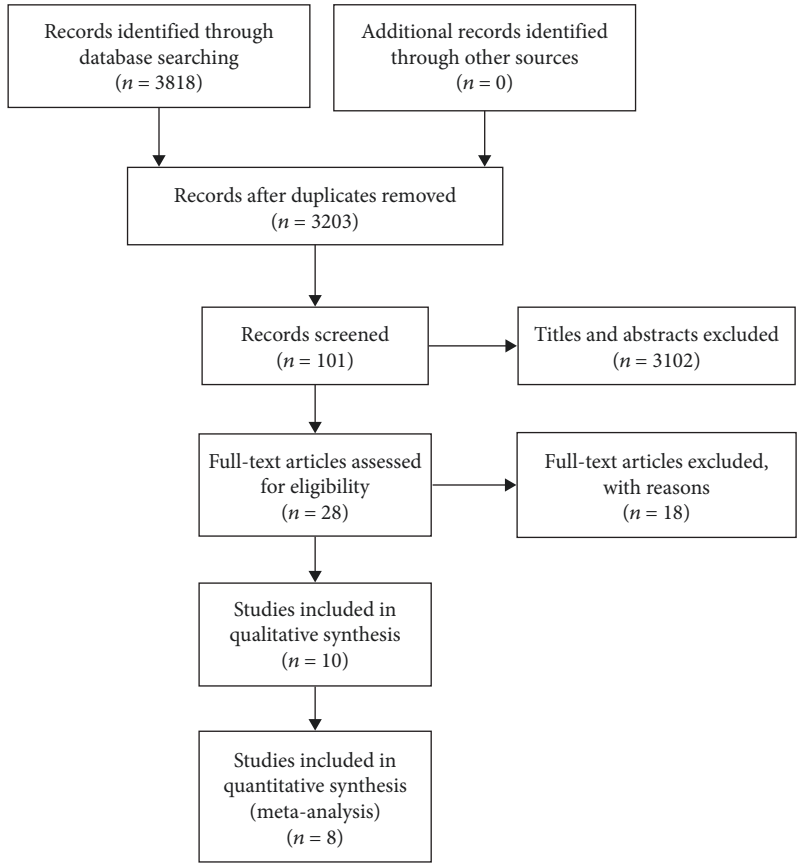

Figure 1: Flow chart of the included studies in the meta-analysis.

were removed because of duplication. A total of 3102 publications were excluded by screening titles and abstracts. Ten studies were included by reviewing the full text, and eight studies were finally identified for our meta-analysis on the basis of the inclusion and exclusion criteria. The article selection process is shown in Figure 1.

3.2. Characteristics of Included Studies. Eight cohort studies were included in our final analysis of 3586 participants [13, 16, 18, 21–25]. Three studies provided information on the sKlotho level and the eGFR (Pearson correlation coefficients or Spearman correlation coefficients) [13, 16, 25], and two studies reported data on sKlotho and an annual decline in eGFR (Pearson correlation coefficients) [21, 24]. Four studies reported data on the sKlotho level and kidney outcomes (HR and its 95% CI) [13, 18, 24, 25]. HR was extrapolated from the Kaplan–Meier curves in one study [23]. Moreover, OR was estimated on the basis of data that were provided in another study [22] because its HR could not be obtained directly or calculated indirectly. Three studies reported correlation coefficients for FGF23 and eGFR [13, 21, 25]. Two studies reported HRs and 95% CIs for FGF23 levels and kidney outcomes [18, 25]. Characteristics of included studies are displayed in Table 1. Quality of the included studies was assessed by the Newcastle–Ottawa Scale for cohort studies. Specific scores that ranged from 4 to 9 are shown in Table 2, and the average score was 7.1.

3.3. Association of sKlotho Levels and the eGFR. One study showed no relationship between sKlotho and the eGFR (the correlation coefficient was not shown in this study) [18], and three studies reported data on the association between sKlotho and the eGFR in the cross-sectional analysis [13, 16, 25]. The remaining four studies did not report any data on sKlotho and the eGFR. All of the three studies demonstrated a positive correlation between sKlotho levels and the eGFR. Meta-analysis showed that the combined Fisher's Z value with the corresponding 95% CI was 0.51 (0.23, 0.79; $P < 0.001$; Figure 2). After inverse Fisher's Z transformation, the summary r and its 95% CI were 0.469 (0.226, 0.658). Our findings suggested that sKlotho levels were positively associated with the eGFR. The random-effect model was used because of significant heterogeneity ($I^2 = 89\%$, $P = 0.0002$, Figure 2). Funnel plots for these studies showed a symmetrical distribution and indicated that there was no publication bias (data not shown). To investigate the source of heterogeneity, we recalculated the combined results by excluding one study each time, and statistical heterogeneity still existed.

Three studies reported data on FGF23 levels and the eGFR, and all of the three studies showed a negative association between FGF23 levels and the eGFR [13, 21, 25]. Our combined Fisher's Z value with its 95% CI was −0.61 (−0.86, −0.36), despite significant heterogeneity ($I^2 = 82\%$, $P = 0.004$, Figure 3), and the calculated summary r and its 95% CI were −0.544 (−0.696, −0.345).

Table 1: Characteristics of the included studies.

First author	Year	Country	Study design	Number	Follow-up period	Average age	Average eGFR(ml/min)	Low versus high sKlotho level	Outcomes	HR and 95% CI
Liu (Ref [25])	2018	China	Cross-sectional Prospective	112	20.1 ± 10.1 months	50.1 ± 14.0	38.2 ± 7.3 22.1 ± 6.3 10.8 ± 2.2	Median sKlotho level	Scr doubling RRT Death	Direct
Bob (Ref [21])	2018	Romania	Cross-sectional Retrospective	63	12 months	58.13 ± 12	65.2 ± 32.5	Overall sKlotho	Δdecline of eGFR	—
Fountoulakis (Ref [23])	2018	UK	Cross-sectional Prospective	101	9 (2-13) years	60 (40-82)	90.7 ± 20.0	Median sKlotho level	50% decline of eGFR Death	Indirect (estimated HR)
Qian (Ref [16])	2018	China	Cross-sectional Prospective	112	1.5 years	64.5 ± 12.7	—	ΔsKlotho level	RRT Cardio-cerebrovascular events	—
Drew (Ref [22])	2017	America	Prospective	2496	3 or 10 years	75 ± 3	73 ± 18	sKlotho quartile level	eGFR decline ≥ 30% or >3 ml/min per year	Indirect (estimated OR)
Kim (Ref [24])	2017	Korea	Prospective	147	32 (12-52) months	56.4 ± 10.8	93.0 ± 23.2	sKlotho tertile level	Annual eGFR decline Albuminuria	Direct
Kim (Ref [13])	2013	Korea	Cross-sectional Prospective	243	29.7 (6.0-62.1) months	45.7 ± 15.7	55.4 ± 36.5	Median sKlotho level	Scr doubling RRT Death	Direct
Seiler (Ref [18])	2013	Germany	Cross-sectional Prospective	312	2.2 ± 0.8 years	65.5 ± 12.1	43.8 ± 15.6	sKlotho tertile level	RRT Death	Direct

Abbreviation: RRT: renal replacement therapy; Scr: serum creatinine; HRs: hazard ratios; CI: confidence interval; eGFR: estimated glomerular filtration rate; Ref: reference.

Table 2: NOS scores of the cohort studies included.

Cohort study	Selection representativeness of the exposed cohort	Selection of the unexposed cohort	Ascertainment of exposure	Outcome of interest not present at start of study	Comparability control for important factor or additional factor*	Outcome assessment	Was follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	Total quality scores
Liu 2018	/	★	★	★	★	★	/	★	6
Bob 2018	/	★	★	★	/	★	/	/	4
Fountoulakis 2018	/	★	★	★	★★	★	★	★	8
Qian 2018	/	★	★	★	★★	★	/	★	7
Drew 2017	★	★	★	★	★★	★	★	★	9
Kim 2016	/	★	★	★	★	★	★	★	7
Kim 2013	/	★	★	★	★★	★	★	★	8
Seiler 2012	/	★	★	★	★★	★	★	★	8

Note: *2 stars could be awarded for this item. Studies that controlled for age or eGFR were awarded one star, respectively. Abbreviation: NOS: Newcastle–Ottawa Scale.

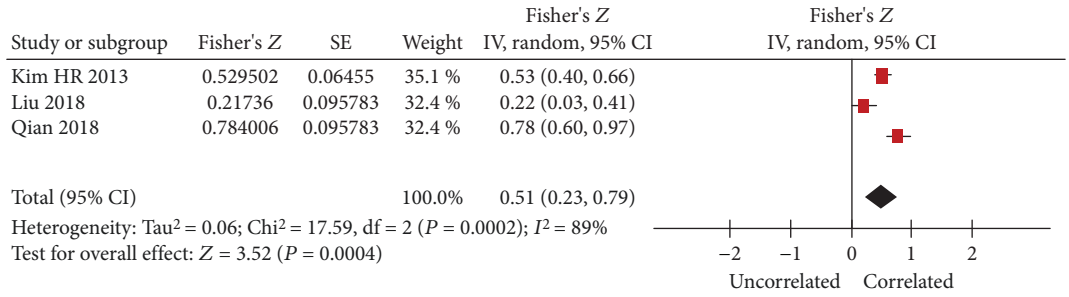

Figure 2: Forest plots of the summary Fisher's Z value with its 95% CIs for the association between sKlotho and eGFR. Summary correlation coefficient (r) and 95% CI were 0.469 (0.226, 0.658) by inverse Fisher's transformation.

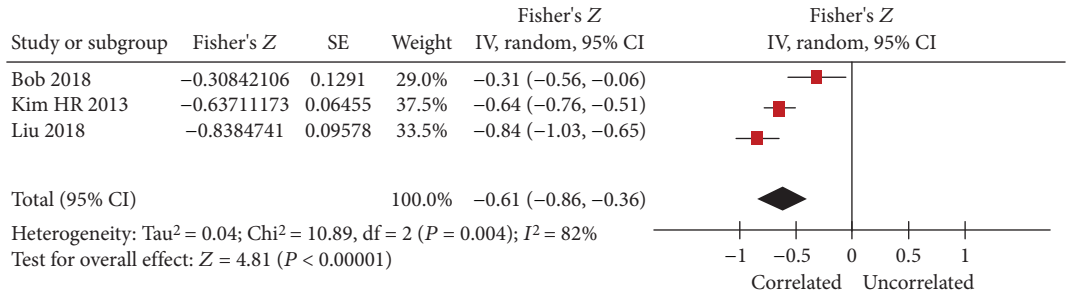

Figure 3: Forest plots of the summary Fisher's Z value with its 95% CIs for the association between FGF23 and eGFR. Summary r and 95% CI were -0.544 (-0.696, -0.345) by inverse Fisher's transformation.

3.4. Association of sKlotho Levels and an Annual Decline in the eGFR. Only two studies reported data on sKlotho levels and an annual decline in the eGFR [21, 24]. In Kim et al.'s study, sKlotho levels were negatively correlated with an annual decline in the eGFR ($r = -0.217$, $P = 0.009$) after adjustment for clinical parameters including baseline eGFR [24]. However, in Bob et al.'s study, sKlotho was positively associated with an annual decline in the eGFR ($r = 0.714$, $P = 0.0004$) and one increase with a standard deviation in the sKlotho level followed by an augmentation with 0.623 standard deviation in the decline of eGFR adjusting for confounding factors [21]. However, high heterogeneity was observed in these two studies ($I^2 = 98\%$, $P < 0.001$) when the data were combined. Due to high heterogeneity and the small number of studies, meta-analysis was not performed further to clear up this confusion.

3.5. Association of sKlotho Levels and Adverse Kidney Outcomes. Of the included eight studies, one study reported that a change in sKlotho levels (ΔsKlotho level) was an independent predictor for an increased risk of RRT after adjusting for confounders [16]. However, one study showed that a high sKlotho level was significantly associated with a rapid decline in the annual eGFR after adjusting for confounding factors [21]. Because HRs or ORs could not be obtained or calculated in these two studies, they were excluded in our meta-analysis. Six studies with 3419 participants were identified as being eligible for our meta-analysis [13, 18, 22–25]. Their HRs or ORs were obtained directly or indirectly after adjustment for possible confounding factors including basal eGFRs. In the six studies, one study showed no relationship between reduced sKlotho levels and increased adverse kidney outcomes (doubling of serum creatinine (Scr) levels or RRT) [18] and the remaining five studies showed a strong correlation [13, 22–25]. With the random-effect model, the pooled HR and its 95% CI were 1.64 (1.19, 2.26; $P = 0.002$; Figure 4), which suggested that low sKlotho levels were significantly associated with increased adverse kidney outcomes. No studies were found to have a significant effect on the total results of this meta-analysis. The distribution of funnel plots was not symmetrical, which suggested that there was publication bias.

There was moderate heterogeneity in the result of the meta-analysis of the included 6 studies ($I^2 = 52\%$, $P = 0.07$, Figure 4). Sensitivity analysis showed that there was no heterogeneity ($I^2 = 1$, $P = 0.40$, Figure 5), and the total combined results were not altered after excluding Drew et al.'s study [22] (pooled HR, 1.78 (1.37, 2.33)). Therefore, Drew et al.'s study was the source of statistical heterogeneity. However, we eventually included this study because of its longest follow-up interval and largest sample size. To further search for the potential causes of significant heterogeneity across the studies, we conducted subgroup meta-analysis on the basis of average age (≥65 years or <65 years), eGFR (≥60 ml/min or <60 ml/min), follow-up interval (≥2 years or <2 years), research region (Asia or other countries), and study quality (score ≥8 or <8). The results of the subgroup meta-analysis are shown in Table 3. Positive associations were still apparent and were significant in all subgroups. There was high heterogeneity regarding the eGFR ($I^2 = 77\%$, $P = 0.04$)

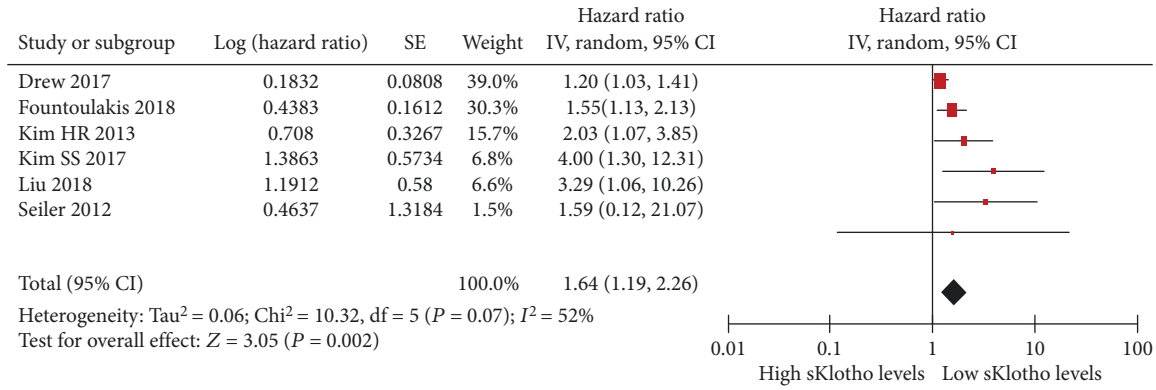

Figure 4: Forest plots of low sKlotho levels and adverse kidney outcomes from the included six studies.

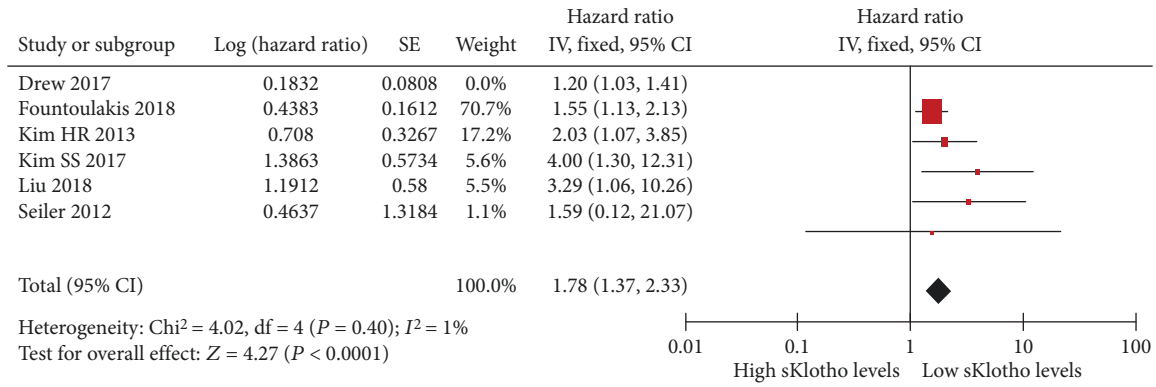

Figure 5: Forest plots of low sKlotho levels and adverse kidney outcomes after removing Drew et al.'s study (2017).

and follow-up interval ($I^2 = 62\%$, $P = 0.05$). There was no statistical heterogeneity regarding the average age ($I^2 = 25\%$, $P = 0.26$), research region ($I^2 = 2\%$,$P = 0.36$), and study quality ($I^2 = 48\%$, $P = 0.15$). Therefore, the eGFR and follow-up interval were thought to be the sources of heterogeneity among the studies.

Three studies showed data on FGF23 levels and adverse kidney outcomes [13, 18, 25]. High FGF23 levels predicted adverse kidney outcomes in two studies [13, 18], but a similar correlation was not observed in our recent study [25]. Because HR or OR was not obtained in Kim et al.'s study [13], this study was removed from our meta-analysis. The overall combined results showed that there was no heterogeneity between the two studies ($I^2 = 0\%$, $P = 0.34$, Figure 6). Thus, meta-analysis was conducted further, and the pooled HR and its 95% CI were 1.96 (1.04, 3.68), indicating that high FGF23 levels were significantly associated with increased adverse kidney outcomes. Although there were a small number of enrolled studies, we believed that the result of the meta-analysis in homogeneous studies was stronger than that of the single original study.

4. Discussion

CKD and its complications are public health issues in the general population. Much effort has been made to screen and identify novel biomarkers for early diagnosis and prognostic estimation in patients with CKD. However, an ideal biomarker is still lacking. The novel antiaging factor sKlotho is a potential biomarker for CKD and has elicited considerable attention in recent years. Levels of sKlotho are primarily generated from the kidney, indicating that there is strong correlation between sKlotho levels and kidney function. Indeed, a number of human studies have suggested that sKlotho levels are not only associated with the state of kidney function but also reflect the extent of kidney injury. Shimamura et al. first reported that sKlotho levels began to decline from CKD stage 2 and continued to decline as CKD progressed [15]. Pavik et al. showed a 1 ml/min decrease in the eGFR accompanied by a 3.2 pg/ml decrease in sKlotho levels [14]. Our recent data also showed that sKlotho levels in CKD were decreased by 75% compared with those in healthy controls [25]. Similar findings have also been found in other human studies with CKD [26–28]. However, several studies have shown conflicting results. In these studies, sKlotho levels were not decreased but increased, or sKlotho levels across CKD stages were not significantly different in patients with CKD [18, 29, 30]. A recent meta-analysis by Wang et al. addressed this discrepancy [17]. In their study, the combined correlation coefficient r (between sKlotho and the eGFR) was 0.35 (0.23, 0.46, $P < 0.05$) [17], which suggested there is a positive correlation between sKlotho and the eGFR [17]. In their meta-analysis, eight studies were

Table 3: Results of subgroup analysis about the association between Klotho and renal outcomes.

Subgroup	Studies	Statistical method	Heterogeneity	Effect estimate	P value
Age	6	HR (IV, fixed, 95% CI)		1.33 (1.16, 1.53)	<0.01
Age ≥ 65	2 (Ref [18, 22])	HR (IV, fixed, 95% CI)	$P = 0.83$; $I^2 = 0\%$	1.20 (1.03, 1.41)	0.02
Age < 65	4 (Ref [13, 23–25])	HR (IV, fixed, 95% CI)	$P = 0.26$; $I^2 = 25\%$	1.79 (1.37, 2.33)	<0.01
eGFR	6	HR (IV, random, 95% CI)		1.81 (1.28, 2.55)	<0.01
eGFR ≥ 60 ml/min	2 (Ref [22, 24])	HR (IV, random, 95% CI)	$P = 0.04$; $I^2 = 77\%$	1.92 (0.61, 6.05)	0.04
eGFR < 60 ml/min	4 (Ref [13, 18, 23, 25])	HR (IV, random, 95% CI)	$P = 0.40$; $I^2 = 1\%$	1.79 (1.37, 2.34)	<0.01
Follow-up interval		HR (IV, random, 95% CI)		1.64 (1.19, 2.26)	<0.01
Follow – up ≥ 2 years	4 (Ref [13, 22–24])	HR (IV, random, 95% CI)	$P = 0.05$; $I^2 = 62\%$	1.56 (1.12, 2.17)	<0.01
Follow – up < 2 years	2 (Ref [18, 25])	HR (IV, random, 95% CI)	$P = 0.61$; $I^2 = 0\%$	2.92 (1.03, 8.28)	0.04
Research region	6	HR (IV, random, 95% CI)		1.64 (1.19, 2.26)	<0.01
Asia	3 (Ref [13, 24, 25])	HR (IV, random, 95% CI)	$P = 0.52$; $I^2 = 0\%$	2.55 (1.55, 4.20)	<0.01
Other countries	3 (Ref [18, 22, 23])	HR (IV, random, 95% CI)	$P = 0.36$; $I^2 = 2\%$	1.27 (1.10, 1.47)	<0.01
Study quality	6	HR (IV, random, 95% CI)		1.64 (1.19, 2.26)	<0.01
High-quality study	4 (Ref [13, 18, 22, 23])	HR (IV, random, 95% CI)	$P = 0.26$; $I^2 = 26\%$	1.37 (1.11, 1.69)	<0.01
Low-quality study	2 (Ref [24, 25])	HR (IV, random, 95% CI)	$P = 0.81$; $I^2 = 0\%$	3.63 (1.63, 8.08)	<0.01

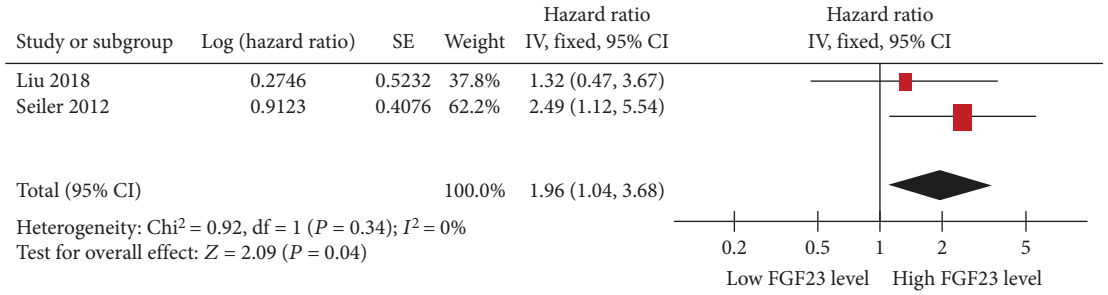

Figure 6: Forest plots of high FGF23 levels and adverse kidney outcomes.

included with 1136 participants, and there was moderate heterogeneity ($I^2 = 68.7\%$, $P = 0.002$) and no publication bias [17]. In line with this previous study, the pooled correlation coefficient r in our study was 0.469 (95% CI: 0.226, 0.658), which indicated that sKlotho levels are linearly related to the eGFR. However, our analysis only included three studies with a small sample size, there was significant heterogeneity, and the strength of our conclusion inevitably was limited. Therefore, our results should be interpreted with caution.

An increasing number of cohort studies have shown that progression of CKD or aggressive loss of the eGFR is significantly associated with sKlotho deficiency. Kim et al. observed that a 10 pg/ml increase was associated with a reduction by 4% in the risk of composite kidney outcomes, including RRT or doubling in Scr levels in patients with CKD stages 1–5 [13]. These authors showed that sKlotho levels below the median value had an increased risk of reaching combined endpoints (HR: 2.03; 95% CI: 1.07, 3.85) [13]. In patients with rapid loss of kidney function, we recently observed that low sKlotho levels were associated with an increased risk of doubling of Scr levels or RRT during follow-up (HR: 3.291; 95% CI: 1.056, 9.823) [25]. In patients with stable kidney function, Drew et al. showed that low sKlotho levels were persistently correlated with adverse kidney outcomes [22]. In this previous study, 2496 participants with a mean eGFR (74 ± 18 ml/min) were enrolled and were followed up for 3 or 10 years. Doubling of sKlotho levels was associated with reduced odds of decline in kidney function for a 30% decline in the eGFR (OR: 0.78; 95% CI: 0.66, 0.93) and for a 3 ml/min per year decline in the eGFR (OR: 0.73; 95% CI: 0.66, 0.99). Overall, low sKlotho levels (below the median value) were associated with a higher risk of reaching combined adverse kidney outcomes (calculated OR: 1.20; 95% CI: 1.03, 1.41) after adjusting for confounders. Because eGFR values were measured at one or two time points at the end of follow-up (3 or 10 years), thus, HR was not reported or calculated in this study. However, some studies provided inconsistent results that sKlotho levels failed to predict progression of CKD [18] and that high sKlotho levels were associated with a rapid decline in kidney function in patients with CKD [21]. Therefore, sKlotho's prognostic significance is still under extensive investigation. In the current study, we found

that low sKlotho levels were associated with increased adverse kidney outcomes, which indicated that sKlotho could be a prognostic biomarker for patients with CKD. Notably, we found significant heterogeneity ($I^2 = 52\%$, $P = 0.07$) in our study. However, significant heterogeneity was no longer present ($I^2 = 1\%$, $P = 0.4$), and the pooled HRs were not altered after the exclusion of Drew et al.'s study [22]. This suggested stability of our meta-analysis results. However, association of sKlotho levels and an annual decline in the eGFR still remains uncertain due to the significant heterogeneity and the small number of studies. Therefore, further studies are needed to resolve the contradictory results.

The mechanism underlying low sKlotho levels increasing the risk of reaching adverse kidney outcomes is multifactorial. Oxidative stress, inflammation, and the renin–angiotensin–aldosterone system are risk factors that promote progression of CKD. Reduced sKlotho levels are also associated with enhanced oxidative stress and inflammation [31, 32]. Interestingly, upregulated Klotho levels facilitate the removal of reactive oxygen species by activating FOXO-mediated manganese superoxide dismutase [33]. Furthermore, upregulated Klotho levels inhibit inflammation by suppressing nuclear factor-κB-mediated inflammatory processes in *in vivo* and *in vitro* studies [34, 35]. Additionally, sKlotho supplementation reduces renal angiotensinogen and angiotensin II levels, followed by the amelioration of renal fibrosis in diabetic and adriamycin nephropathy [36, 37]. Moreover, sKlotho therapy suppresses renal fibrosis by targeting several fibrotic signaling pathways, including TGFβ-1/Smads and WNT/β-catenin signaling [37–39]. In our previous studies, sKlotho treatment inhibited renal fibrosis via suppression of endoplasmic reticulum stress or epithelial-mesenchymal transition [40, 41]. Because of the pleiotropic beneficial activities of sKlotho, it is a novel kidney-protective factor and treatment target for renal fibrosis [42]. Deficiency of sKlotho makes the kidney vulnerable to attacks from oxidative stress, ischemia, and inflammation, and this in turn aggravates kidney function. Therefore, loss of sKlotho is implicated in the development and progression of CKD, which is supported by our results.

FGF23 is primarily secreted by osteocytes and osteoblasts and was newly identified as a regulator of phosphorylation by forming a complex with Klotho [43]. FGF23 levels are increased in the early stages of CKD, and this even precedes the elevation of the parathyroid hormone and phosphate levels [43, 44]. As a compensatory response, elevated FGF23 levels are mostly ascribed to an increase in phosphate burden, and this maintains normal phosphorus levels in early CKD [44, 45]. As the eGFR continues to decline in the course of CKD, this compensatory mechanism fails to maintain phosphorus homeostasis, and this in turn leads to higher FGF23 levels [46]. Therefore, FGF23 is elevated in patients in the early stage of CKD, and this trend increases as CKD progresses because of persistent retention of serum phosphorus levels [47, 48]. FGF23 levels are inversely correlated with the eGFR, and they are emerging as an early biomarker for CKD in recent years [49–51]. Our findings are consistent with these previous findings that FGF23 is negatively correlated with the eGFR. Accumulating evidence has shown that increased FGF23 levels are strongly associated with an increased risk of adverse kidney outcomes or mortality in prospective studies on patients with CKD [13, 18, 52, 53]. This finding indicates that FGF23 may also have a prognostic value in these patients. However, our recent study showed conflicting results [25]. To address this issue, we also assessed the prognostic value of FGF23 in our included studies in the meta-analysis. We found that the pooled HR was 1.96 (95% CI: 1.04, 3.68) from two studies with 412 participants and there was no heterogeneity ($I^2 = 0\%$, $P = 0.34$). Another study reported that a 10 pg/ml increase in FGF23 levels was related to a 4% increase of risk of adverse kidney outcomes and supported the strong correlation between the high FGF23 levels and increased risk of adverse kidney outcomes [13]. Taken together, our results showed that high FGF23 levels predicted adverse kidney outcomes in CKD patients with sKlotho loss. Therefore, an increase in FGF23 levels with a decrease in sKlotho levels may be associated with the development and progression of CKD [49]. However, the small number of enrolled studies inevitably reduced the power of our conclusion, and definite data are still lacking.

Above all, our meta-analysis of published longitudinal studies suggested that sKlotho levels were positively correlated with the eGFR and that a low sKlotho level predicted poor kidney outcomes in patients with CKD in adjusted analyses. These findings provide evidence for sKlotho as a potential biomarker for early detection and prognostic evaluation of CKD.

Our study has several limitations. First, there was significant heterogeneity. Sensitivity analysis showed that exclusion of Drew et al.'s study completely eliminated heterogeneity [22]. However, we eventually included this study because it represented the largest sample size and longest follow-up period among the included studies. We believe that the eGFR and follow-up interval, at least partly, are the sources of observed heterogeneity according to the results of subgroup meta-analysis. sKlotho is mainly produced by the kidney, and the state of kidney function affects sKlotho levels. Patients with an eGFR < 60 ml/min are always accompanied by lower sKlotho levels, and this condition is prone to suffering from more adverse outcomes. With regard to the follow-up interval, CKD progresses slowly under standard medical care and the risk of reaching outcomes is associated with the duration of follow-up. This means that the occurrence of adverse outcomes may depend on follow-up interval to some extent. Heterogeneity may also be caused by differences in patients' characteristics, including research region, sex, race, assay use, and other confounders. Moreover, the studies included are relatively few, especially the number of studies for FGF23 and renal outcomes, and the sample size is relatively small. Metaregression is not conducted further to investigate the source of heterogeneity. Therefore, these may have led to underestimation of our combined results, although the random-effect model was used for our analysis. Second, there was significant publication bias. Our meta-analysis included studies that were restricted to English publications. There may have been a few studies with negative results, a small sample size, or written in other languages that were not published. This inevitably resulted in publications

bias. Third, some HRs in two studies were not reported in two studies [22, 23]. Instead, we had to extrapolate HR from the Kaplan–Meier curves [23] or calculate ORs according to the original data [22], and estimated HR or OR is less reliable. Fourth, the reference value for sKlotho has not been determined, and sKlotho levels are ranging from 326.4 to 2.44 ng/ml. The cut-off values of sKlotho are variable, and the method used for the Klotho assay varied in this meta-analysis. Moreover, the sKlotho level may be regulated by some drugs or the acute inflammatory process, which were not entirely excluded in the included studies. The fluctuations may have lessened the power of our results to some extent.

In conclusion, our meta-analysis shows that sKlotho levels are positively correlated with the eGFR. Moreover, low sKlotho levels are associated with an increased risk of reaching adverse kidney outcomes in patients with CKD. Our findings support the assumption that sKlotho could be used as a novel indicator for early diagnosis and prognostic assessment of CKD, despite the limitations discussed above. Prospective studies with larger sample sizes are still required to confirm our conclusions.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Qi-feng Liu and Jian-Ming Ye designed the study. Li-xia Yu and Qiang Sun collected and extracted the data. Sha-sha Li and Jian-hua Feng performed the data analysis. Qi-feng Liu wrote the manuscript. All authors approved the data and final version of the submitted manuscript. Qi-feng Liu and Li-xia Yu contributed equally to this work.

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References

[1] M. Kuro-o, Y. Matsumura, H. Aizawa et al., "Mutation of the mouse klotho gene leads to a syndrome resembling ageing," *Nature*, vol. 390, no. 6655, pp. 45–51, 1997.

[3] L. D. Quarles, "Fibroblast growth factor 23 and α-Klotho co-dependent and independent functions," *Current Opinion in Nephrology and Hypertension*, vol. 28, no. 1, pp. 1–25, 2019.

[4] M. Kuro-o, "The Klotho proteins in health and disease," *Nature Reviews Nephrology*, vol. 15, no. 1, pp. 27–44, 2019.

[5] Y. Kato, E. Arakawa, S. Kinoshita et al., "Establishment of the anti-Klotho monoclonal antibodies and detection of Klotho protein in kidneys," *Biochemical and Biophysical Research Communications*, vol. 267, no. 2, pp. 597–602, 2000.

[6] G. D. Dalton, J. Xie, S. W. An, and C. L. Huang, "New insights into the mechanism of action of soluble Klotho," *Frontiers in Endocrinology*, vol. 8, p. 323, 2017.

[7] K. Lim, A. Groen, G. Molostvov et al., "α-Klotho expression in human tissues," *The Journal of Clinical Endocrinology & Metabolism*, vol. 100, no. 10, pp. E1308–E1318, 2015.

[8] K. Takeshita, T. Fujimori, Y. Kurotaki et al., "Sinoatrial node dysfunction and early unexpected death of mice with a defect of klotho gene expression," *Circulation*, vol. 109, no. 14, pp. 1776–1782, 2004.

[9] M. C. Hu, M. Shi, J. Zhang et al., "Renal production, uptake, and handling of circulating αKlotho," *Journal of the American Society of Nephrology*, vol. 27, no. 1, pp. 79–90, 2015.

[10] K. Lindberg, R. Amin, O. W. Moe et al., "The kidney is the principal organ mediating Klotho effects," *Journal of the American Society of Nephrology*, vol. 25, no. 10, pp. 2169–2175, 2014.

[11] Z. Liu, H. Zhou, X. Chen et al., "Relationship between cFGF23/Klotho ratio and phosphate levels in patients with chronic kidney disease," *International Urology and Nephrology*, vol. 51, no. 3, pp. 503–507, 2019.

[12] J. K.-C. Ng and P. K.-T. Li, "Chronic kidney disease epidemic: how do we deal with it?," *Nephrology*, vol. 23, pp. 116–120, 2018.

[13] H. R. Kim, B. Y. Nam, D. W. Kim et al., "Circulating α-Klotho levels in CKD and relationship to progression," *American Journal of Kidney Diseases*, vol. 61, no. 6, pp. 899–909, 2013.

[14] I. Pavik, P. Jaeger, L. Ebner et al., "Secreted Klotho and FGF23 in chronic kidney disease stage 1 to 5: a sequence suggested from a cross-sectional study," *Nephrology Dialysis Transplantation*, vol. 28, no. 2, pp. 352–359, 2013.

[15] Y. Shimamura, K. Hamada, K. Inoue et al., "Serum levels of soluble secreted α-Klotho are decreased in the early stages of chronic kidney disease, making it a probable novel biomarker for early diagnosis," *Clinical and Experimental Nephrology*, vol. 16, no. 5, pp. 722–729, 2012.

[16] J. Qian, J. Zhong, M. Yan et al., "Circulating α-Klotho is related to plasma aldosterone and its follow-up change predicts CKD progression," *Kidney and Blood Pressure Research*, vol. 43, no. 3, pp. 836–846, 2018.

[17] Q. Wang, W. Su, Z. Shen, and R. Wang, "Correlation between soluble α-Klotho and renal function in patients with chronic kidney disease: a review and meta-analysis," *BioMed Research International*, vol. 2018, Article ID 9481475, 12 pages, 2018.

[18] S. Seiler, M. Wen, H. J. Roth et al., "Plasma Klotho is not related to kidney function and does not predict adverse outcome in patients with chronic kidney disease," *Kidney International*, vol. 83, no. 1, pp. 121–128, 2013.

[19] J. F. Tierney, L. A. Stewart, D. Ghersi, S. Burdett, and M. R. Sydes, "Practical methods for incorporating summary time-to-event data into meta-analysis," *Trials*, vol. 8, no. 1, p. 16, 2007.

[20] A. Stang, "Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses," *European Journal of Epidemiology*, vol. 25, no. 9, pp. 603–605, 2010.

[21] F. Bob, A. Schiller, R. Timar et al., "Rapid decline of kidney function in diabetic kidney disease is associated with high soluble Klotho levels," *Nefrologia*, vol. 39, no. 3, pp. 250–257, 2019.

[22] D. A. Drew, R. Katz, S. Kritchevsky et al., "Association between soluble Klotho and change in kidney function: the health aging and body composition study," *Journal of the American Society of Nephrology*, vol. 28, no. 6, pp. 1859–1866, 2017.

[23] N. Fountoulakis, G. Maltese, L. Gnudi, and J. Karalliedde, "Reduced levels of anti-ageing hormone Klotho predict renal function decline in type 2 diabetes," *The Journal of Clinical Endocrinology & Metabolism*, vol. 103, no. 5, pp. 2026–2032, 2018.

[24] S. S. Kim, S. H. Song, I. J. Kim et al., "Decreased plasma α-Klotho predict progression of nephropathy with type 2 diabetic patients," *Journal of Diabetes and its Complications*, vol. 30, no. 5, pp. 887–892, 2016.

[25] Q. F. Liu, J. M. Ye, L. X. Yu et al., "Plasma s-Klotho is related to kidney function and predicts adverse renal outcomes in patients with advanced chronic kidney disease," *Journal of Investigative Medicine*, vol. 66, no. 3, pp. 669–675, 2018.

[26] V. M. Brandenburg, M. E. Kleber, M. G. Vervloet et al., "Soluble Klotho and mortality: the Ludwigshafen Risk and Cardiovascular Health Study," *Atherosclerosis*, vol. 242, no. 2, pp. 483–489, 2015.

[27] S. Rotondi, M. Pasquali, L. Tartaglione et al., "Soluble α-Klotho serum levels in chronic kidney disease," *International Journal of Endocrinology*, vol. 2015, Article ID 872193, 8 pages, 2015.

[28] H. Sakan, K. Nakatani, O. Asai et al., "Reduced renal α-Klotho expression in CKD patients and its effect on renal phosphate handling and vitamin D metabolism," *PLoS One*, vol. 9, no. 1, article e86301, 2014.

[29] A. Scholze, Y. Liu, L. Pedersen et al., "Soluble α-Klotho and its relation to kidney function and fibroblast growth factor-23," *The Journal of Clinical Endocrinology & Metabolism*, vol. 99, no. 5, pp. E855–E861, 2014.

[30] H. Sugiura, K. Tsuchiya, and K. Nitta, "Circulating levels of soluble α-Klotho in patients with chronic kidney disease," *Clinical and Experimental Nephrology*, vol. 15, no. 5, pp. 795-796, 2011.

[31] Y. Haruna, N. Kashihara, M. Satoh et al., "Amelioration of progressive renal injury by genetic manipulation of *Klotho* gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 7, pp. 2331–2336, 2007.

[32] H. J. Oh, B. Y. Nam, M. J. Lee et al., "Decreased circulating Klotho levels in patients undergoing dialysis and relationship to oxidative stress and inflammation," *Peritoneal Dialysis International*, vol. 35, no. 1, pp. 43–51, 2015.

[33] M. Yamamoto, J. D. Clark, J. V. Pastor et al., "Regulation of oxidative stress by the anti-aging hormone Klotho," *Journal of Biological Chemistry*, vol. 280, no. 45, pp. 38029–38034, 2005.

[34] Y. Zhao, S. Banerjee, N. Dey et al., "Klotho depletion contributes to increased inflammation in kidney of the *db*/*db* mouse model of diabetes via RelA (serine)^{536} phosphorylation," *Diabetes*, vol. 60, no. 7, pp. 1907–1916, 2011.

[35] P. Buendía, J. Carracedo, S. Soriano et al., "Klotho prevents NFκB translocation and protects endothelial cell from senescence induced by uremia," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 70, no. 10, pp. 1198–1209, 2015.

[36] T. Takenaka, T. Inoue, T. Miyazaki et al., "Klotho suppresses the renin-angiotensin system in adriamycin nephropathy," *Nephrology Dialysis Transplantation*, vol. 32, no. 5, pp. 791–800, 2016.

[37] T. Takenaka, H. Kobori, T. Miyazaki et al., "Klotho protein supplementation reduces blood pressure and renal hypertrophy in db/db mice, a model of type 2 diabetes," *Acta Physiologica*, vol. 225, no. 2, article e13190, 2019.

[38] S. Doi, Y. Zou, O. Togao et al., "Klotho inhibits transforming growth factor-β1 (TGF-β1) signaling and suppresses renal fibrosis and cancer metastasis in mice," *Journal of Biological Chemistry*, vol. 286, no. 10, pp. 8655–8665, 2011.

[39] L. Zhou, Y. Li, D. Zhou, R. J. Tan, and Y. Liu, "Loss of Klotho contributes to kidney injury by derepression of Wnt/β-catenin signaling," *Journal of the American Society of Nephrology*, vol. 24, no. 5, pp. 771–785, 2013.

[40] Q. F. Liu, J. M. Ye, Z. Y. Deng, L. X. Yu, Q. Sun, and S. S. Li, "Ameliorating effect of Klotho on endoplasmic reticulum stress and renal fibrosis induced by unilateral ureteral obstruction," *Iranian Journal of Kidney Diseases*, vol. 9, no. 4, pp. 291–297, 2015.

[41] Q. F. Liu, J. M. Ye, L. X. Yu et al., "Klotho mitigates cyclosporine A (CsA)-induced epithelial-mesenchymal transition (EMT) and renal fibrosis in rats," *International Urology and Nephrology*, vol. 49, no. 2, pp. 345–352, 2017.

[42] D. Zou, W. Wu, Y. He, S. Ma, and J. Gao, "The role of Klotho in chronic kidney disease," *BMC Nephrology*, vol. 19, no. 1, p. 285, 2018.

[43] H. Kurosu, Y. Ogawa, M. Miyoshi et al., "Regulation of fibroblast growth factor-23 signaling by Klotho," *Journal of Biological Chemistry*, vol. 281, no. 10, pp. 6120–6123, 2006.

[44] J. Chudek, P. Koce ak, A. Owczarek et al., "Fibroblast growth factor 23 (FGF23) and early chronic kidney disease in the elderly," *Nephrology Dialysis Transplantation*, vol. 29, no. 9, pp. 1757–1763, 2014.

[45] T. Isakova, P. Wahl, G. S. Vargas et al., "Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease," *Kidney International*, vol. 79, no. 12, pp. 1370–1378, 2011.

[46] R. G. Erben and O. Andrukhova, "FGF23-Klotho signaling axis in the kidney," *Bone*, vol. 100, pp. 62–68, 2017.

[47] T. Larsson, U. L. F. Nisbeth, Ö. Ljunggren, H. Jüppner, and K. B. Jonsson, "Circulating concentration of FGF-23 increases as renal function declines in patients with chronic kidney disease, but does not change in response to variation in phosphate intake in healthy volunteers," *Kidney International*, vol. 64, no. 6, pp. 2272–2279, 2003.

[48] J. X. Li, G. Q. Yu, and Y. Z. Zhuang, "Impact of serum FGF23 levels on blood pressure of patients with chronic kidney disease," *European Review for Medical and Pharmacological Sciences*, vol. 22, no. 3, pp. 721–725, 2018.

[49] X. Lu and M. C. Hu, "Klotho/FGF23 axis in chronic kidney disease and cardiovascular disease," *Kidney Diseases*, vol. 3, no. 1, pp. 15–23, 2017.

[50] H. Li, Z. Yu, L. Gan, L. Peng, and Q. Zhou, "Serum NGAL and FGF23 may have certain value in early diagnosis of CIN," *Renal Failure*, vol. 40, no. 1, pp. 547–553, 2018.

[51] S. Y. Liu, D. D. Zhang, Y. F. Wu et al., "Fibroblast growth factor-23 may serve as a novel biomarker for renal osteodystrophy progression," *International Journal of Molecular Medicine*, vol. 43, no. 1, pp. 535–546, 2019.

[52] T. Isakova, X. Cai, J. Lee et al., "Longitudinal FGF23 trajectories and mortality in patients with CKD," *Journal of the American Society of Nephrology*, vol. 29, no. 2, pp. 579–590, 2018.

[53] I. Jialal, F. Camacho, B. Nathoo, P. Tam, R. Pahwa, and G. G. Wu, "Fibroblast growth factor 23 predicts mortality and end-stage renal disease in a Canadian Asian population with chronic kidney disease," *Nephron*, vol. 137, no. 3, pp. 190–196, 2017.

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Research Article

Does Whole-Blood Neutrophil Gelatinase-Associated Lipocalin Stratify Acute Kidney Injury in Critically Ill Patients?

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Purpose. To analyse the capacity of whole-blood NGAL (wbNGAL) to stratify AKI in critically ill patients with and without sepsis. *Methods*. Whole-blood NGAL was measured with a point-of-care device at admission and 48 hours later in patients admitted to a general ICU. Patients were classified by the AKIN and KDIGO classifications at admission and 24 and 48 hours. We performed an ROC curve analysis. wbNGAL values at admission were compared in patients with sepsis and septic shock. *Results*. The study included 100 consecutively admitted patients (40 female) with mean age 59.1 ± 17.8 years. Thirty-three patients presented AKI at admission, and 10 more developed it in the next 48 h. Eighteen patients had AKI stage 3, 14 of them at admission. Nine patients required renal replacement therapy. According to KDIGO at admission, wbNGAL values were 78 μg/L (60-187) in stage 0 ($n = 67$), 263 μg/L (89-314) in stage 1 ($n = 8$), 484 μg/L (333-708) in stage 2 ($n = 11$), and 623 μg/L (231-911) in stage 3 ($n = 14$), $p = 0.0001$ for trend. Ten patients did not complete 48 hours of study: 6 of 10 were discharged (initial wbNGAL 130 μg/L (60-514)) and 4 died (773 μg/L (311-1010)). The AUROC curve of wbNGAL to predict AKI was 0.838 (95% confidence interval 0.76-0.92, $p = 0.0001$), with optimal cut-off value of 178 μg/L (sensitivity 76.7%, specificity 78.9%, $p < 0.0001$). At admission, twenty-nine patients had sepsis, of whom 20 were in septic shock. wbNGAL concentrations were 81 μg/L (60-187) in patients without sepsis, 481 (247-687) in those with sepsis, and 623.5 μg/L (361-798) in the subgroup of septic shock ($p < 0.0001$). *Conclusions*. Whole-blood NGAL concentration at ICU admission was a good stratifier of AKI in critically ill patients. However, wbNGAL concentrations were higher in septic patients irrespective of AKI occurrence.

1. Introduction

Acute kidney injury (AKI) is a disease often diagnosed in intensive care unit (ICU) patients. AKI incidence can be as high as 24% depending on its definition [1]. ICU-related AKI is associated with an in-hospital mortality that can reach 60% [2, 3]. Therefore, early AKI detection is crucial to prevent or stop the natural course of renal dysfunction and improve its morbi-mortality. Serum creatinine is the gold standard biomarker in all AKI definitions [4–6]. However, creatinine is a late biomarker for AKI, as values peak after 24-48 hours of renal injury. Hence, several biomarkers have been proposed for early AKI detection [7, 8].

Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein first isolated from specific granules of human leukocytes [9]. NGAL was found elevated in bacterial infections when compared to viral infections [10]. NGAL exists as a monomeric form of 25 kDa, a homodimer linked by a disulphide bridge of 45 kDa and a heterodimer with matrix metalloproteinase-9 (MMP-9, gelatinase) with an intermolecular disulphide bridge of 135 kDa [11]. NGAL is also formed in other cells apart from leukocytes. In response to several injuries, NGAL is expressed in kidney, hepatic, or epithelial cells [11–13]. In the kidneys, NGAL is freely filtered by the glomerulus and reabsorbed in the proximal tubule [14]. After a tubular injury, NGAL is overexpressed in the diseased

endothelium [15–17]. In this setting, tubular reabsorption is reduced, and blood and urine NGAL concentrations increase. In AKI, NGAL increase is observed 24-48 hours earlier than plasma creatinine peak [18, 19]. However, the specificity of NGAL to detect AKI could be limited by overexpression in other tissues [20]. This could be relevant in critically ill patients, in whom sepsis could promote NGAL release from tissues other than renal epithelium [21, 22]. NGAL has been described as a predictor of AKI and the need of renal replacement therapies (RRT) in patients admitted to general intensive care units (ICU) [21–24]. However, as a systematic review by Hjortrup et al. [24] pointed out, the NGAL role is not fully understood: literature shows that NGAL might predict AKI with a wide range AUROC, from 0.54 to 0.98. The aims of this study were (1) to evaluate the capacity of whole-blood NGAL at ICU admission to predict AKI development and (2) to analyse the effect of sepsis on its predictive capacity.

2. Methods

2.1. Patients. The study protocol was approved by the Ethic Committee Board at Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). We obtained informed consent from participants or their guardians. The study prospectively included 100 patients consecutively admitted during 8 months (June 2010-February 2011) in a general ICU. Inclusion criteria were age over 18 years old and the expected ICU stay of at least 48 hours. Patients were excluded if they had already been admitted in the hospital for more than 24 hours, had any degree of preexisting chronic kidney disease, or were not expected to survive for at least 24 hours due to a nonreversible clinical condition. Physicians attending patients were blinded to NGAL results throughout the study.

2.2. Clinical and Laboratory Data Collection. Clinical data included admission diagnosis, demographics, ICU severity scores, haemodynamic parameters, and urine output. Patients were initially classified following the AKIN definition [5] and KDIGO [6] definition for AKI. Both definitions were based on serum creatinine and urine output obtained since admission to 6 hours later, at 24 and 48 hours of admission. Baseline serum creatinine was taken from patients' preadmission records whenever possible and used to estimate eGFR before ICU admission using the Cockcroft–Gault formula. According to standard criteria [25], patients were also classified as having sepsis and septic shock.

NGAL was measured at admission and 24 and 48 hours in EDTA-anticoagulated whole-blood using the Triage® NGAL Test (Alere Diagnostics, formerly Inverness Medical Innovations). All samples were analysed in the same batch to avoid between-batch variability.

2.3. Statistical Analysis. SPSS® version 18 (SPSS Inc., Chicago, IL) was used. Variables with normal distribution are reported as mean ± standard deviation and were compared with Student's t-test or one-way analysis of variance. Variables with non-Gaussian distribution are reported as median and interquartile range (IQR) and were compared with the Mann–Whitney U or Kruskal-Wallis tests. Categorical data are reported as percentage and were compared by the chi-square test or Fisher exact test. Reporting of results followed the STARD (Standards for Reporting Diagnostic Accuracy Studies) statement. Whole-blood NGAL predictive values were evaluated by receiver operating characteristic (ROC) curve analysis. We defined an area under the ROC (AUROC) curve of 0.60-0.69 as poor, 0.70-0.79 as fair, 0.80-0.89 as good, and 0.90-1.00 as excellent in terms of predictive value. A $p < 0.05$ was considered significant.

3. Results

3.1. Clinical Characteristics. We recruited 100 consecutive patients fulfilling the admission criteria (Figure 1). Ten of them did not complete the 48 h follow-up due to discharge (6 cases) or death (4 cases). Mean age was 59.1 ± 17.8 years. 60% of cases were male. The causes of ICU admission were medical 54% (respiratory 26%, cardiovascular 7%), postsurgical care 39% (gastrointestinal 21%, neurosurgery 12%), and miscellaneous 7%. At ICU admission, twenty-nine were septic, and twenty of them had septic shock. ICU length of stay was 10.3 ± 9.6 days, and ICU mortality was 22%.

3.2. Whole-Blood NGAL and AKI. Forty-three patients presented AKI, 33 at admission (8 stage 1, 11 stage 2, and 14 stage 3) and 10 more within 48 h of the ICU stay; 4 of the latest group of 10 developed renal failure (stage 3). Nine patients required renal replacement therapies. wbNGAL values were 78 μg/L (IQR 60-187 μg/L) in non-AKI patients and 263 μg/L (IQR 89-314 μg/L), 484 μg/L (IQR 333-708 μg/L), and 623 μg/L (IQR 231-911 μg/L) in those with stage 1, stage 2, and stage 3, respectively ($p = 0.0001$ for trend) (Figure 2). Four of 33 patients with AKI at admission were diagnosed of AKI solely because of urine output criterion (one patient had stage 1, one patient had stage 2, and two patients had stage 3); 10 of 33 patients were diagnosed of AKI based on changes in sCr, and the remaining 19 patients were diagnosed based in both sCr and drop of urine output. The incidence and severity of AKI were the same when AKIN classification was applied within the first 48 h.

In the group of 6 patients discharged before 24 h, admission wbNGAL and plasma creatinine were of 130 μg/L (IQR 60-514) and 78 μmol/L (IQR 54-123), respectively; whereas in the 4 patients who died, wbNGAL was of 773 μg/L (IQR 311-1010) and plasma creatinine 165 μmol/L (IQR 59-577). We did not find statistical differences in wbNGAL or serum creatinine between subgroups of patients who were discharged or passed away.

Whole-blood NGAL values were predictive of AKI both at admission and within 48 h of the ICU stay (Supplemental Table 1). The area under the ROC curve (AUROC) of wbNGAL for AKI prediction within 48 hours of ICU admission was 0.838 (95% confidence interval (CI) 0.760-0.917, $p = 0.0001$). The wbNGAL optimal cut-off for AKI within 48 h of ICU admission was 178 μg/L, with sensitivity of 76.7% and specificity of 78.9% (Figure 3). The AUROC of admission serum creatinine for AKI within the first 48 h

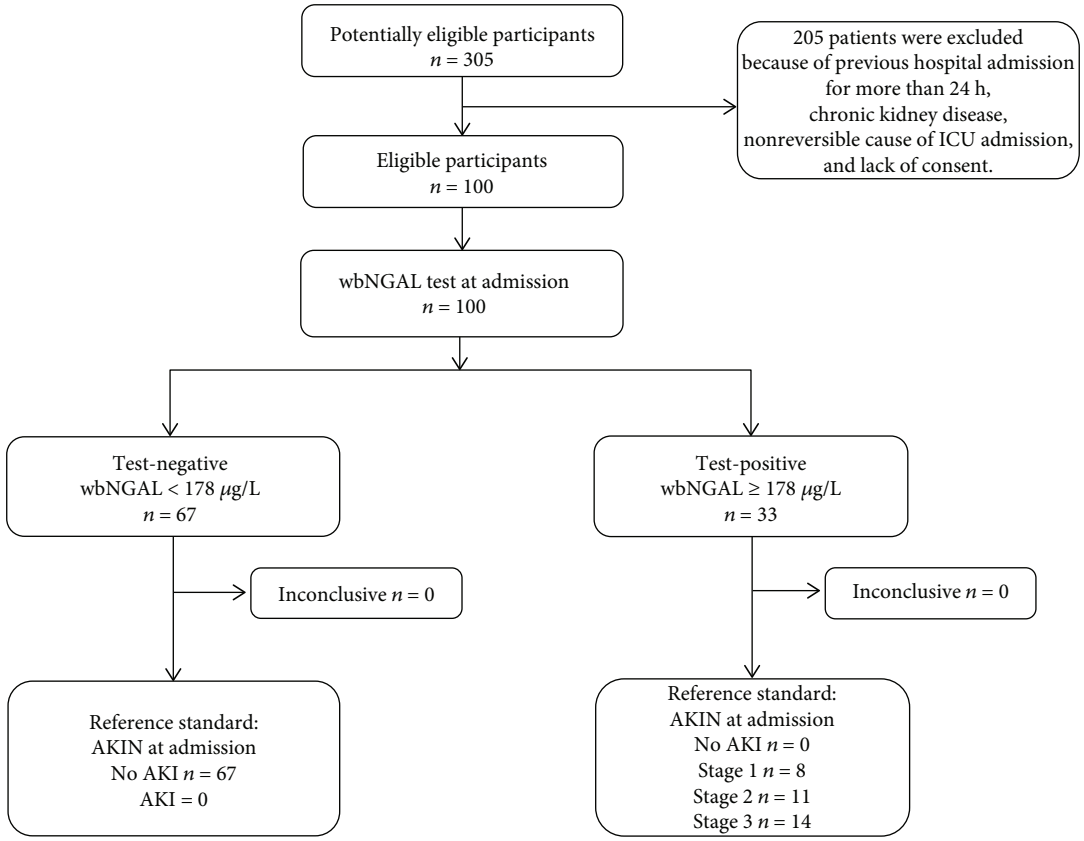

Figure 1: Diagram of whole-blood NGAL to predict AKI, study enrolment and inclusion/exclusion criteria.

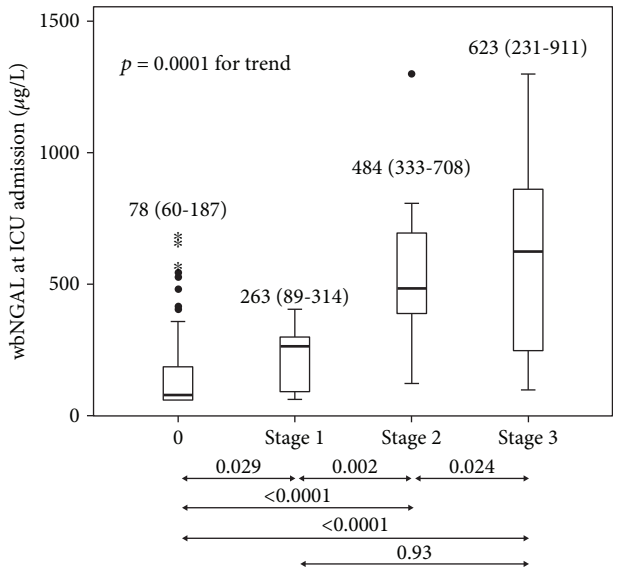

Figure 2: Boxplot comparing whole-blood NGAL (wbNGAL) concentrations (μg/L) and KDIGO score at admission. Boxplots indicate the median and 25th and 75th percentiles. Whiskers indicate the 5th and 95th percentiles. Statistical significance (p) comparing wbNGAL (μg/L) with KDIGO categories is given at the bottom of the figure.

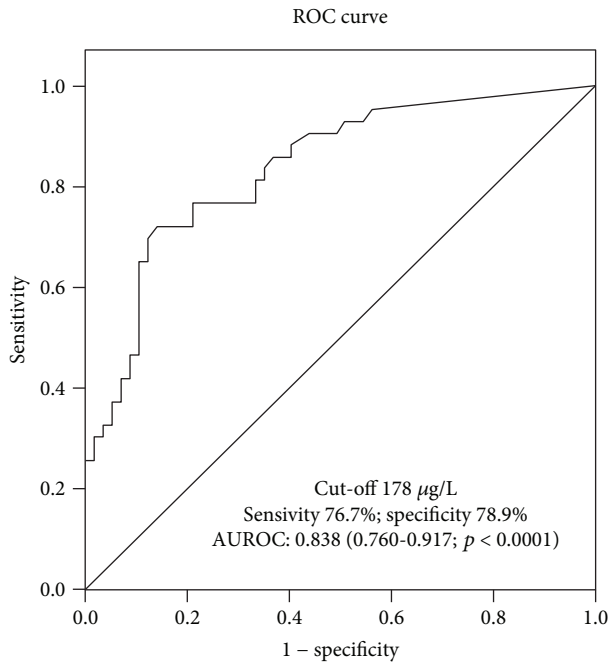

Figure 3: ROC curve for whole-blood NGAL value at ICU admission to predict AKI. Diagnostic and overall accuracies given with sensitivity, specificity, and 95% confidence interval compared with gold standard KDIGO classification.

Table 1: Clinical characteristics of the study patients depending on the cut-off obtained for AKI prediction.

	<178 μg/L	≥178 μg/L	p value
n	55	45	—
Age (years)	54.9 ± 18.5	64 ± 15.5	0.009
Male	37 (67)	23 (51)	0.075
Characteristics at ICU admission			
SAPS II	37.8 ± 14.3	43.15 ± 15.5	0.095
APACHE II	15 ± 6.9	18 ± 8.2	0.059
SOFA	5.2 ± 2.7	7.7 ± 3.6	<0.0001
AKI admission (%)	6 (10.9)	27 (15.6)	<0.0001
KDIGO stage 3 at admission (%)	2 (3.6)	12 (26.7)	0.001
RRT admission (%)	2 (3.6)	3 (6.7)	0.400
Creatinine clearance (mL/min)	109 ± 58	57 ± 52	<0.0001
Cockcroft–Gault	104 ± 47	58 ± 38	<0.0001
Characteristics during ICU stay			
Mechanical ventilation (%)	46 (82)	36 (80)	0.410
Vasopressor requirement (%)	6 (11)	22 (49)	<0.0001
ICU length of stay (days)	11.1 ± 9.3	9.4 ± 10	0.360
ICU mortality (%)	10 (18.2)	12 (26.7)	0.210
Sepsis	3 (5.5)	26 (57.8)	0.007
Septic shock	2 (3.6)	18 (40)	<0.0001
AKI development (%)	2 (3.6)	6 (13.3)	0.070
KDIGO stage 3 development (%)	2 (3.6)	16 (35.6)	<0.0001
RRT after admission (%)	0 (0)	4 (8.9)	0.038
RRT total (%)	2 (3.6)	7 (15.6)	0.040

Values expressed as either % per column or mean ± standard deviation. p: value of statistical significance. SAPS II: Simplified Acute Physiology Score II; APACHE II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment score; AKI: acute kidney injury; KDIGO: Improving Global Outcomes AKI group classification; RRT: Renal replacement therapies; ICU: intensive care unit.

of ICU admission was 0.904 (95% CI 0.841-0.967, $p = 0.0001$). There were no statistical differences between wbNGAL and plasma creatinine AUROC.

Forty-five patients had wbNGAL > 178 μg/L. They were more likely to be older, have higher SOFA at admission, have higher incidence of AKI development, stage 3, sepsis, or septic shock, and have requirement of vasopressor drugs and renal replacement therapies during the ICU stay (Table 1). Six of eighteen patients without AKI at admission but wbNGAL > 178 μg/L developed AKI within the next 48 h. Accordingly, wbNGAL identified at admission extra 14.6% of AKI patients not diagnosed by the serum creatinine criterion.

3.3. Whole-Blood NGAL and Sepsis. wbNGAL concentrations were 81 μg/L (IQR 60-187) and 481 μg/L (IQR 247-681) in 71 patients without sepsis and 29 patients with sepsis, respectively ($p < 0.0001$) (Figure 4). wbNGAL was 623.5 μg/L (IQR 361-798) in those 20 of 29 septic patients who also had shock. The incidence of AKI in sepsis and septic shock was 28.6% and 65%, respectively.

Serial measurement of wbNGAL did not improve AKI prediction in septic patients compared to nonseptic patients (data not shown). In nonseptic patients, AKI concentrations appeared dependent on AKI status: 62 μg/L (IQR 60-99) in non-AKI and 297 μg/L (IQR 123-502) in AKI patients, $p < 0.0001$. However, when comparing wbNGAL in septic patients with (632 μg/L (IQR 344-1060)) or without (414 μg/L (IQR 214-552)) AKI, there was no

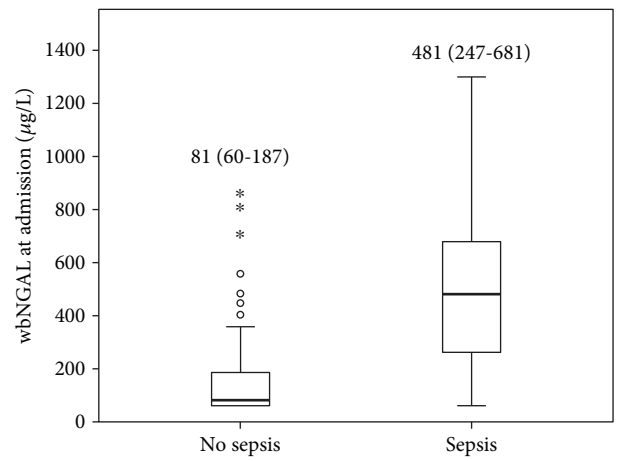

Figure 4: Whole-blood NGAL concentrations (μg/L) according to the presence of sepsis. Boxplots indicate the median and 25th and 75th percentiles of wbNGAL in ng/L. Whiskers indicate the 5th and 95th percentiles. Statistical significance (p). Patients without sepsis $n = 71$; patients with sepsis $n = 29$.

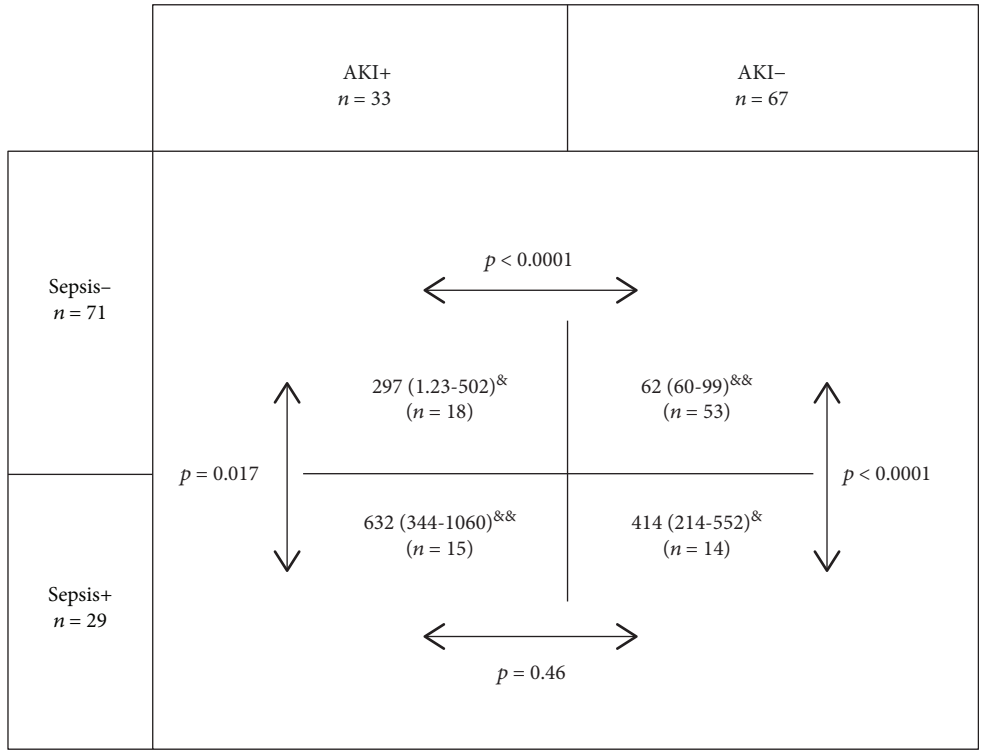

Figure 5: Distribution of admission whole-blood NGAL (μg/L) values at ICU admission depending on AKI and sepsis. AKI+ vs. AKI- and sepsis+ vs. sepsis- represents the presence or absence of either AKI or sepsis upon admission, respectively. Values show median and percentiles 25-75. p represents the statistical intragroup differences. wbNGAL values given in μg/L. [&]Statistical difference between subgroups of nonseptic AKI vs. septic non-AKI patients ($p = 0.676$). [&&]Statistical difference between subgroups of nonseptic non-AKI vs. septic and AKI patients ($p < 0.0001$). AKI: acute kidney injury.

statistical difference ($p = 0.46$). Septic patients with and without AKI presented higher wbNGAL than those with the same renal status without sepsis ($p < 0.0001$ for both comparisons). wbNGAL values in septic non-AKI patients were undistinguishable of those of nonseptic AKI patients ($p = 0.676$). Nine of the 11 non-AKI patients with wbNGAL higher than the cut-off >178 μg/L presented sepsis (Figure 5). In our study, there were 20 patients with septic shock: 3 had no AKI (median wbNGAL of 481 μg/L, IQR 142-481 μg/L), 2 had stage 1 (251, IQR 234-269 μg/L), 4 had stage 2 (562, IQR 421-681 μg/L), and 11 had stage 3 (685, IQR 526-1130 μg/L).

4. Discussion

The main finding of our study is that admission wbNGAL is a good stratifier of AKI within 48 hours of ICU admission in a heterogeneous group of critically ill patients (AUROC 0.838 (95% CI 0.760-0.917, $p < 0.0001$)). The cut-off value higher than 178 μg/L defines a group of patients with higher severity and higher probability of developing AKI. However, NGAL concentrations were affected by sepsis status irrespective of the AKI presence ($p = 0.46$). Thus, NGAL measures are not useful to evaluate kidney function in patients with sepsis.

Some studies suggested that NGAL in blood or urine could be a useful biomarker of AKI [26]. Most of those initial studies were done in patients after cardiac surgery [7, 27], in context of contrast-induced nephropathy [28], kidney transplantation [18], or chronic kidney disease [29]. Fewer studies were done in general critically ill population, both paediatric [30, 31] and adult patients [19, 21, 32, 33]. There is still debate whether NGAL is a good predictor of AKI in general ICU patients [24]. Our AUROC of wbNGAL is significantly higher than that found by Parikh et al. [18] in the postoperative care in cardiac surgery and by Haase et al. [22] in a paediatric critically ill population. In our study, wbNGAL stratified AKI severity, showing increasing concentrations with an increased KDIGO stage from median values of 71 μg/L in stage 1 and 186 μg/L in stage 2 to 381 μg/L in stage 3 (Figure 2). This increasing pattern in line with the severity of AKI has also been described by other investigators in paediatric and adult patients [24, 25]. Although wbNGAL concentrations in non-AKI patients are similar to those found by Singer et al. [25], AKI severity categories were lower in our study. In view of our results, we would suggest clinicians to consider wbNGAL as a complementary tool in patients at AKI risk or receiving nephrotoxic drugs. Despite NGAL's promising results in general ICU population, the AKIKI [34], ELAIN [35], and the most recent IDEAL-ICU [36] trials showed that wbNGAL combined with KDIGO staging did not improve the timing of renal replacement therapies or patient's prognosis.

In our cohort, the AUROC curve of wbNGAL for AKI prediction showed an optimal cut-off value of 178 μg/L. Our cut-off is close to the ones described in other studies, around 150 μg/L [22, 27, 37]. In critically ill patients, the

exact time when acute tubular damage occurs is often unknown. The differences in AUROC described in literature could also be explained by their study designs. Unlike our study, those set up in cardiac surgery or radiocontrast administration had an exact time of a potential onset of renal injury.

We found significant differences in admission wbNGAL between the groups of patients with AKI and those without. We also identified a subgroup of patients who presented high levels of wbNGAL with no increase of creatinine. This subgroup could be considered false positives or, in our opinion, a subgroup of patients who may represent subclinical AKI [22]. Patients with stage 1 showed relatively low wbNGAL concentrations within the group of patients with AKI. This finding could be attributable to treated reversible causes of AKI, like hypovolemia or hypotension. Nickolas et al. [38] described low levels of urine NGAL in patients with prerenal azotaemia. Those patients inadequately resuscitated after a prerenal azotaemia had increasing urinary NGAL concentrations. Similar to our study, this is a clinically relevant finding. Timely treatment of patients with subclinical AKI or stage 1 could avoid AKI progression to failure and tubular damage.

NGAL is a protein upregulated after a tubular injury [39]. However, it can also be produced by other organs like the liver or lung [7] or in different inflammatory situations [40]. In our study, the serial measurement of wbNGAL did not improve AKI prediction in septic patients compared to nonseptic patients. No statistical differences were found between ROC curves obtained at admission and 48 h later; both ROC curves produced very similar cut-offs (Supplemental Table 1). These results concur with those from Bagshaw et al. [41], which showed that peak plasma NGAL did not perform better to predict AKI in septic than in nonseptic patients. This is clinically relevant because single sampling at admission decreases costs, and it is easier to implement in daily routine.

wbNGAL values were higher in patients with sepsis and much higher in those with septic shock. These data strongly suggest that wbNGAL not only is a good predictor of AKI but also can be considered a good severity score in patients with inflammatory status. Although Mishra et al. [27] showed that plasma NGAL was independent of inflammatory markers like C-reactive protein, Zappitelli et al. [31] reported that plasma and urine NGAL concentrations at ICU admission were higher in patients in which AKI was due to sepsis than in those in which it was due to nonseptic causes. Like in our study, these authors found that serial NGAL measurement did not add predictive power to NGAL concentrations at admission. Other articles also suggested that the inflammatory status [24] or septic shock could influence wbNGAL concentrations [22], and this could be found regardless of the presence of AKI [42]. Kim et al. [43] described that wbNGAL was significantly higher in septic patients with AKI regardless of their levels of procalcitonin. In our subgroup of 20 patients with septic shock, 3 of them had no AKI, 2 stage 1, 4 stage 2, and 11 stage 3, with median wbNGAL of 481 μg/L, 251 μg/L, 562 μg/L, and 685 μg/L, respectively. These data favour the hypothesis that inflammatory status could increase the level of wbNGAL and could be misleadingly interpreted as a tubular damage when it has not yet occurred. In these cases, urinary NGAL, which is not submitted to the influence of inflammatory mediators, could have better performance in AKI prediction. Besides, differentiation of monomeric and dimeric plasma NGAL isoforms could be crucial to recognise the NGAL concentration secondary to renal damage or inflammation [44]. Our data also suggest that NGAL may be a biomarker of illness severity. In an article by Shapiro et al. [8], NGAL was a good predictor of septic shock in a panel of multiple biomarkers and correlated with survival. Wang et al. also described that high wbNGAL independently predicted mortality and multiple organ dysfunction syndrome in sepsis and septic shock [45].

On the other hand, a study set up in the emergency department by Wang et al. [46] suggested that combined NGAL and TIMP-1 (tissue inhibitor of matrix metalloproteinases-1, a cell cycle arrest biomarker for AKI) was useful for the diagnosis and risk stratification of patients with AKI, including those who also presented with sepsis. Although cell cycle arrest (CCA) biomarkers are still under evaluation in different clinical settings, their sole predictive power and risk stratification appears to be higher than that exhibited by NGAL. Since CCA biomarkers were presented in Sapphire study [47], there has been an increasing number of studies in paediatric [48] and adult critical care populations, with clinical implementation in rapid response teams [49] and KDIGO care bundles [50, 51]. Besides, unlike NGAL, CCA biomarkers are not determined by sepsis [52, 53].

Our study has some limitations. First of all, the sample size was small, and the study was performed in a single centre. Secondly, we recruited a cohort that might not represent an average ICU population. We aimed to study the AKI incidence in a population with ideally normal baseline renal function. AKI is a common complication of many nonrenal hospitalisations [54–56]. We purposefully excluded patients with a background of chronic disease and/or admitted in the hospital for more than 24 hours prior to ICU admission. However, this is also the main strength of our study, because our unique cohort of patients were not under the effect of intrahospital risk factors for AKI that could have acted as confounding variables. Finally, the point-of-care test used to analyse wbNGAL was not able to differentiate between NGAL isoforms, which could have helped to explain the role of inflammation in wbNGAL concentrations.

5. Conclusions

Our study showed that whole-blood NGAL concentrations at ICU admission stratified AKI in adult critically ill patients. Nonetheless, wbNGAL concentrations increased by sepsis status irrespective of AKI occurrence. Thus, NGAL measures should be avoided to evaluate kidney function in patients with sepsis.

Data Availability

The data used to support the findings of this study are restricted by the Hospital de la Santa Creu i Sant Pau's Ethic Committee Board in order to protect patient privacy. Data

are available from authors (Dr. Antoni Jordi Betbese Roig, ajbetbese@santpau.cat) for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

KN received congress fees from Abbot to present the preliminary results of this study at ESICM 2011.

Authors' Contributions

Data collection was done by KN, JB, and MC. The study design, data analysis, and interpretation were independently performed by AJB, JO-Ll, and MC.

Acknowledgments

The study sponsor (Abbot) was responsible for providing the portable device (Triage® NGAL Test (Alere Diagnostics, formerly Inverness Medical Innovations)). The authors would like to thank the staff in the ICU and biochemistry departments who generously participated in sample processing and analysis and whose work is essential to the completion of this study.

Supplementary Materials

Supplemental Table 1: diagnostic and overall accuracy of wbNGAL for AKI prediction, RRT at admission, and sepsis at admission. *(Supplementary Materials)*

References

[1] S. Uchino, J. A. Kellum, R. Bellomo et al., "Acute renal failure in critically ill patients: a multinational, multicenter study," *Journal of the American Medical Association*, vol. 294, no. 7, pp. 813–818, 2005.

[2] VA/NIH Acute Renal Failure Trial Network, P. M. Palevsky, J. H. Zhang et al., "Intensity of renal support in critically ill patients with acute kidney injury," *The New England Journal of Medicine*, vol. 359, no. 1, pp. 7–20, 2008.

[3] W. Silvester, R. Bellomo, and L. Cole, "Epidemiology, management, and outcome of severe acute renal failure of critical illness in Australia," *Critical Care Medicine*, vol. 29, no. 10, pp. 1910–1915, 2001.

[4] R. Bellomo, C. Ronco, J. A. Kellum, R. L. Mehta, P. Palevsky, and Acute Dialysis Quality Initiative workgroup, "Acute renal failure – definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group," *Critical Care*, vol. 8, no. 4, article R204, 2004.

[5] R. L. Mehta, J. A. Kellum, S. V. Shah et al., "Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury," *Critical Care*, vol. 11, no. 2, article R31, 2007.

[6] Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group, "KDIGO clinical practice guideline for acute kidney injury," *Kidney International Supplement*, vol. 2, pp. 1–138, 2012.

[7] A. Haase-Fielitz, R. Bellomo, P. Devarajan et al., "Novel and conventional serum biomarkers predicting acute kidney injury in adult cardiac surgery-a prospective cohort study," *Critical Care Medicine*, vol. 37, no. 2, pp. 553–560, 2009.

[8] N. I. Shapiro, S. Trzeciak, J. E. Hollander et al., "A prospective, multicenter derivation of a biomarker panel to assess risk of organ dysfunction, shock, and death in emergency department patients with suspected sepsis," *Critical Care Medicine*, vol. 37, no. 1, pp. 96–104, 2009.

[9] L. Kjeldsen, A. H. Johnsen, H. Sengelov, and N. Borregaard, "Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase," *The Journal of Biological Chemistry*, vol. 268, no. 14, pp. 10425–10432, 1993.

[10] S. Y. Xu, K. Pauksen, and P. Venge, "Serum measurements of human neutrophil lipocalin (HNL) discriminate between acute bacterial and viral infections," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 55, no. 2, pp. 125–131, 1995.

[11] J. B. Cowland, O. E. Sorensen, M. Sehested, and N. Borregaard, "Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1β, but not by TNF-α," *The Journal of Immunology*, vol. 171, no. 12, pp. 6630–6639, 2003.

[12] J. B. Cowland and N. Borregaard, "Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans," *Genomics*, vol. 45, no. 1, pp. 17–23, 1997.

[13] J. Mishra, K. Mori, Q. Ma et al., "Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin," *Journal of the American Society of Nephrology*, vol. 15, no. 12, pp. 3073–3082, 2004.

[14] K. M. Schmidt-Ott, K. Mori, J. Y. Li et al., "Dual action of neutrophil gelatinase-associated lipocalin," *Journal of the American Society of Nephrology*, vol. 18, no. 2, pp. 407–413, 2007.

[15] K. Leelawat, S. Narong, J. Wannaprasert, and S. Leelawat, "Serum NGAL to clinically distinguish cholangiocarcinoma from benign biliary tract diseases," *International Journal of Hepatology*, vol. 2011, Article ID 873548, 6 pages, 2011.

[16] A. A. Zabron, V. M. Horneffer-van der Sluis, C. A. Wadsworth et al., "Elevated levels of neutrophil gelatinase-associated lipocalin in bile from patients with malignant pancreatobiliary disease," *The American Journal of Gastroenterology*, vol. 106, no. 9, pp. 1711–1717, 2011.

[17] T. M. Eagan, J. K. Damås, T. Ueland et al., "Neutrophil gelatinase-associated lipocalin: a biomarker in COPD," *Chest*, vol. 138, no. 4, pp. 888–895, 2010.

[18] C. R. Parikh, A. Jani, J. Mishra et al., "Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation," *American Journal of Transplantation*, vol. 6, no. 7, pp. 1639–1645, 2006.

[19] D. N. Cruz, M. de Cal, F. Garzotto et al., "Plasma neutrophil gelatinase-associated lipocalin is an early biomarker for acute kidney injury in an adult ICU population," *Intensive Care Medicine*, vol. 36, no. 3, pp. 444–451, 2010.

[20] Q. Liu and M. Nilsen-Hamilton, "Identification of a new acute phase protein," *Journal of Biological Chemistry*, vol. 270, no. 38, pp. 22565–22570, 1995.

[21] E. D. Siew, L. B. Ware, T. Gebretsadik et al., "Urine neutrophil gelatinase-associated lipocalin moderately predicts acute kidney injury in critically ill adults," *Journal of the American Society of Nephrology*, vol. 20, no. 8, pp. 1823–1832, 2009.

[22] M. Haase, P. Devarajan, A. Haase-Fielitz et al., "The outcome of neutrophil gelatinase-associated lipocalin-positive subclinical acute kidney injury. A multicenter pooled analysis of prospective studies," *Journal of the American College of Cardiology*, vol. 57, no. 17, pp. 1752–1761, 2011.

[23] P. Kümpers, C. Hafer, A. Lukasz et al., "Serum neutrophil gelatinase-associated lipocalin at inception of renal replacement therapy predicts survival in critically ill patients with acute kidney injury," *Critical Care*, vol. 14, no. 1, article R9, 2010.

[24] P. B. Hjortrup, N. Haase, M. Wetterslev, and A. Perner, "Clinical review: predictive value of neutrophil gelatinase-associated lipocalin for acute kidney injury in intensive care patients," *Critical Care*, vol. 17, no. 2, p. 211, 2013.

[25] M. Singer, C. S. Deutschman, C. W. Seymour et al., "The third international consensus definitions for sepsis and septic shock (sepsis-3)," *Journal of the American Medical Association*, vol. 315, no. 8, pp. 801–810, 2016.

[26] M. Haase, R. Bellomo, P. Devarajan, P. Schlattmann, A. Haase-Fielitz, and NGAL Meta-analysis Investigator Group, "Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis," *American Journal of Kidney Diseases*, vol. 54, no. 6, pp. 1012–1024, 2009.

[27] J. Mishra, C. Dent, R. Tarabishi et al., "Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery," *The Lancet*, vol. 365, no. 9466, pp. 1231–1238, 2005.

[28] R. Hirsch, C. Dent, H. Pfriem et al., "NGAL is an early predictive biomarker of contrast-induced nephropathy in children," *Pediatric Nephrology*, vol. 22, no. 12, pp. 2089–2095, 2007.

[29] D. Bolignano, G. Coppolino, A. Romeo, A. Lacquaniti, and M. Buemi, "Neutrophil gelatinase-associated lipocalin levels in chronic haemodialysis patients," *Nephrology*, vol. 15, no. 1, pp. 23–26, 2010.

[30] D. S. Wheeler, P. Devarajan, Q. Ma et al., "Serum neutrophil gelatinase-associated lipocalin (NGAL) as a marker of acute kidney injury in critically ill children with septic shock," *Critical Care Medicine*, vol. 36, no. 4, pp. 1297–1303, 2008.

[31] M. Zappitelli, K. K. Washburn, A. A. Arikan et al., "Urine neutrophil gelatinase-associated lipocalin is an early marker of acute kidney injury in critically ill children: a prospective cohort study," *Critical Care*, vol. 11, no. 4, article R84, 2007.

[32] K. Makris, N. Markou, E. Evodia et al., "Urinary neutrophil gelatinase-associated lipocalin (NGAL) as an early marker of acute kidney injury in critically ill multiple trauma patients," *Clinical Chemistry and Laboratory Medicine*, vol. 47, no. 1, pp. 79–82, 2009.

[33] J.-M. Constantin, E. Futier, S. Perbet et al., "Plasma neutrophil gelatinase-associated lipocalin is an early marker of acute kidney injury in adult critically ill patients: a prospective study," *Journal of Critical Care*, vol. 25, no. 1, pp. 176.e1–176.e6, 2010.

[34] S. Gaudry, D. Hajage, F. Schortgen et al., "Initiation strategies for renal-replacement therapy in the intensive care unit," *The New England Journal of Medicine*, vol. 375, no. 2, pp. 122–133, 2016.

[35] A. Zarbock, J. A. Kellum, C. Schmidt et al., "Effect of early vs delayed initiation of renal replacement therapy on mortality in critically ill patients with acute kidney injury the ELAIN randomized clinical trial," *Journal of the American Medical Association*, vol. 315, no. 20, pp. 2190–2199, 2016.

[36] S. Barbar, R. Clere-Jehl, A. Bourredjem et al., "Timing of renal-replacement therapy in patients with acute kidney injury and sepsis," *The New England Journal of Medicine*, vol. 379, no. 15, pp. 1431–1442, 2018.

[37] C. L. Dent, Q. Ma, S. Dastrala et al., "Plasma neutrophil gelatinase-associated lipocalin predicts acute kidney injury, morbidity and mortality after pediatric cardiac surgery: a prospective uncontrolled cohort study," *Critical Care*, vol. 11, no. 6, article R127, 2007.

[38] T. L. Nickolas, M. J. O'Rourke, J. Yang et al., "Sensitivity and specificity of a single emergency department measurement of urinary neutrophil gelatinase-associated lipocalin for diagnosing acute kidney injury," *Annals of Internal Medicine*, vol. 148, no. 11, pp. 810–819, 2008.

[39] K. Damman, D. J. van Veldhuisen, G. Navis, A. A. Voors, and H. L. Hillege, "Urinary neutrophil gelatinase associated lipocalin (NGAL), a marker of tubular damage, is increased in patients with chronic heart failure," *European Journal of Heart Failure*, vol. 10, no. 10, pp. 997–1000, 2008.

[40] B. S. Nielsen, N. Borregaard, J. R. Bundgaard, S. Timshel, M. Sehested, and L. Kjeldsen, "Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases," *Gut*, vol. 38, no. 3, pp. 414–420, 1996.

[41] S. M. Bagshaw, M. Bennett, M. Haase et al., "Plasma and urine neutrophil gelatinase-associated lipocalin in septic versus non-septic acute kidney injury in critical illness," *Intensive Care Medicine*, vol. 36, no. 3, pp. 452–461, 2010.

[42] J. Vanmassenhove, G. Glorieux, N. Lameire et al., "Influence of severity of illness on neutrophil gelatinase-associated lipocalin performance as a marker of acute kidney injury: a prospective cohort study of patients with sepsis," *BMC Nephrology*, vol. 16, no. 1, p. 18, 2015.

[43] H. Kim, M. Hur, D. N. Cruz, H. W. Moon, and Y. M. Yun, "Plasma neutrophil gelatinase-associated lipocalin as a biomarker for acute kidney injury in critically ill patients with suspected sepsis," *Clinical Biochemistry*, vol. 46, no. 15, pp. 1414–1418, 2013.

[44] N. J. Glassford, A. G. Schneider, S. Xu et al., "The nature and discriminatory value of urinary neutrophil gelatinase-associated lipocalin in critically ill patients at risk of acute kidney injury," *Intensive Care Medicine*, vol. 39, no. 10, pp. 1714–1724, 2013.

[45] B. Wang, G. Chen, J. Zhang, J. Xue, Y. Cao, and Y. Wu, "Increased neutrophil gelatinase associated lipocalin is associated with mortality and multiple organ dysfunction syndrome in severe sepsis and septic shock," *Shock*, vol. 44, no. 3, pp. 234–238, 2015.

[46] M. Wang, Q. Zhang, X. Zhao, G. Dong, and C. Li, "Diagnostic and prognostic value of neutrophil gelatinase-associated lipocalin, matrix metalloproteinase-9, and tissue inhibitor of matrix metalloproteinases-1 for sepsis in the emergency department: an observational study," *Critical Care*, vol. 18, no. 6, p. 634, 2014.

[47] K. Kashani, A. al-Khafaji, T. Ardiles et al., "Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury," *Critical Care*, vol. 17, no. 1, article R25, 2013.

[48] J. H. Westhoff, B. Tönshoff, S. Waldherr et al., "Urinary tissue inhibitor of metalloproteinase-2 (TIMP-2) • insulin-like growth factor-binding protein 7 (IGFBP7) predicts adverse outcome in pediatric acute kidney injury," *PLoS One*, vol. 10, no. 11, article e0143628, 2015.

[49] L. M. Rizo-Topete, M. H. Rosner, and C. Ronco, "Acute kidney injury risk assessment and the nephrology rapid response team," *Blood Purification*, vol. 43, no. 1-3, pp. 82–88, 2017.

[50] M. Meersch, C. Schmidt, A. Hoffmeier et al., "Prevention of cardiac surgery-associated AKI by implementing the KDIGO guidelines in high risk patients identified by biomarkers: the PrevAKI randomized controlled trial," *Intensive Care Medicine*, vol. 43, no. 11, pp. 1551–1561, 2017.

[51] I. Göcze, D. Jauch, M. Götz et al., "Biomarker-guided intervention to prevent acute kidney injury after major surgery: the prospective randomized BigpAK Study," *Annals of Surgery*, vol. 267, no. 6, pp. 1013–1020, 2018.

[52] P. M. Honore, H. B. Nguyen, M. Gong et al., "Urinary tissue inhibitor of metalloproteinase-2 and insulin-like growth factor-binding protein 7 for risk stratification of acute kidney injury in patients with sepsis," *Critical Care Medicine*, vol. 44, no. 10, pp. 1851–1860, 2016.

[53] M. Cuartero, J. Ballús, J. Sabater et al., "Cell-cycle arrest biomarkers in urine to predict acute kidney injury in septic and non-septic critically ill patients," *Annals of Intensive Care*, vol. 7, no. 1, p. 92, 2017.

[54] S. H. Hou, D. A. Bushinsky, J. B. Wish, J. J. Cohen, and J. T. Harrington, "Hospital-acquired renal insufficiency: a prospective study," *The American Journal of Medicine*, vol. 74, no. 2, pp. 243–248, 1983.

[55] K. Nash, A. Hafeez, and S. Hou, "Hospital-acquired renal insufficiency," *American Journal of Kidney Diseases*, vol. 39, no. 5, pp. 930–936, 2002.

[56] H. E. Wang, P. Muntner, G. M. Chertow, and D. G. Warnock, "Acute kidney injury and mortality in hospitalized patients," *American Journal of Nephrology*, vol. 35, no. 4, pp. 349–355, 2012.

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Research Article

Evaluation of the Diagnostic Potential of uPAR as a Biomarker in Renal Biopsies of Patients with FSGS

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Minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) are primary glomerulopathies leading to proteinuria, known as podocytopathies, which share syndromic and morphological similarities. Morphological similarity occurs in cases of FSGS in which the sclerotic lesion was not sampled in renal biopsy, due to the focal nature of the disease. Differentiating these entities is very important, especially in cases of suspected FSGS but with sclerotic lesion not sampled, as they are diseases that apparently have different pathogenic mechanisms and prognosis. The difference in uPAR expression in situ among these two entities may be related to a distinct molecular mechanism involved in pathogenesis. Thus, finding biomarkers involved in the pathogenesis and that can also help in differential diagnosis is very relevant. The aim of this work was to evaluate the potential of urokinase-type plasminogen activator receptor (uPAR) as a biomarker in renal biopsies of patients with podocytopathies ($n = 38$). Immunohistochemistry showed that FSGS ($n = 22$) had increased uPAR expression in podocytes compared with both the MCD group ($n = 16$; $p = 0.0368$) and control group ($n = 21$; $p = 0.0076$). ROC curve ($p = 0.008$) showed that this biomarker has 80.95% of specificity in biopsies of patients with FSGS. Therefore, uPAR presented a high specificity in cases of podocytopathies associated with sclerosis and it can be considered a potential biomarker for FSGS.

1. Introduction

Glomerular diseases are among the leading causes of end-stage renal disease worldwide. The main clinical feature of patients with glomerulopathies is nephrotic syndrome (NS), which is characterized by nephrotic range proteinuria (>3.5 g/day), hypoalbuminemia (serum albumin < 3 g/dl), hyperlipidemia (serum cholesterol > 200 mg/dl), and edema, affecting both adults and children [1]. Podocytes are highly specialized epithelial cells with a unique architecture that covers the outer surfaces of glomerular capillaries, supporting the glomerular filtration barrier [2, 3]. Podocyte injury may lead to effacement of their extensions, the foot process, leading to proteinuria [4].

Focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD) are podocytopathies, characterized primarily by changes in podocytes [1] and have clinical and morphological similarities, sometimes making it difficult to distinguish between them. Morphological similarity occurs specially in cases of nonsampled FSGS in renal biopsy, as

sclerosis in this disease, by definition, is a focal finding: not all glomeruli are affected [5]. Thus, it is very important to find biomarkers involved in pathogenesis of these entities and that, in addition, can help in diagnosis [6, 7].

Some authors distinguish these entities based on differences in their clinical presentations and histological characteristics [8, 9], as opposed to others who believe they are different manifestations of the same progressive disease, in which FSGS would be an advanced stage [10]. Pathogenesis of these entities is controversial, but it seems to be related to structural and/or molecular podocyte changes, and some proteins have also been associated with renal damage and proteinuria [1]. In this way, uPAR/suPAR has been proposed to have a role in FSGS pathogenesis [11–15].

uPAR is a membrane-bound 45-55 kDa protein with three domains (DI, DII, and DIII) linked to glycosylphosphatidylinositol (GPI). It is found in several immunologically active cells, as well as in podocytes [16]. Once bound to its ligand, it can promote cell adhesion, migration, and cell proliferation disorders [17]. In podocytes, it was observed that uPAR is able to activate $\alpha v\beta 3$ integrin promoting cell mobility and activation of small GTPases, such as Cdc42 and Rac1, thus allowing contraction of podocytes, which acquire motility and consequently foot process effacement, as well as development of proteinuria [18].

uPAR is released from cell surface in a soluble form, suPAR, which can be found in several body fluids, including plasma, urine, saliva, and cerebrospinal fluid at different levels and with similar functions to uPAR [16].

Due to the common recurrence of FSGS after transplantation, it is possible that circulating factors may be involved in the pathogenesis of this disease [19]. suPAR was thought to be this possible circulating factor, as circulating suPAR could activate $\alpha v\beta 3$ integrin similarly to membrane-bound uPAR in podocytes [11].

There are literature controversies concerning the role of uPAR/suPAR as a biomarker of FSGS as suPAR levels are also increased in other glomerular diseases [20–23]. However, a growing body of evidence suggests a role of suPAR as a scaring factor in FSGS [11, 12, 19, 24, 25].

suPAR has been proposed to have a role in FSGS pathogenesis although this is debated and unclear. Despite this, there is no study evaluating the potential of uPAR staining in renal biopsy as a way of differentiating FSGS from MCD. So, we decided to explore the role of uPAR in differentiating FSGS and MCD.

2. Methods

2.1. Patients. Thirty-eight cases of podocytopathies were selected, comprising patients with FSGS ($n = 22$) and MCD ($n = 16$) from the Nephropathology Service of General Pathology Discipline of Federal University of Triângulo Mineiro (UFTM), Uberaba, Minas Gerais State, Brazil. The groups were divided as follows: (a) the FSGS group, defined by the presence of segmental sclerosis (increase in mesangial matrix) and, in electron microscopy, foot process effacement, and (b) the MCD group, defined by foot process effacement as an isolated finding in electron microscopy. The control group ($n = 21$) was composed of autopsy kidneys from patients whose death was not related to renal or infectious diseases.

The ethics and research committee of Federal University of Triângulo Mineiro approved this study with the number 1.715.838.

2.2. Renal Histopathology. Renal specimens were evaluated by direct immunofluorescence, light, and electron microscopy similar to the Huang et al. technique [26]. For direct immunofluorescence, immunoglobulins IgG, IgM, and IgA; kappa and lambda light chains; complement fractions C3 and C1q; and fibrinogen were detected by fluorescein isothiocyanate- (FITC-) conjugated antibodies (Dako, Copenhagen, Denmark) on frozen tissues. For light microscopy, paraffin sections were stained with hematoxylin and eosin, sirius red, silver methenamine stain (PAMS), and Masson's trichrome. For electron microscopy, in brief, tissue was fixed in 2.5% Karnovsky +0.2% ruthenium red and latter fixed in osmium tetroxide 2% and then dehydrated in graded alcohols and acetone solutions and embedded in Epon 812. Ultrathin sections were cut with 60 nm thickness and placed on nickel grids. Then, ultrathin sections were stained with uranyl acetate and examined with a transmission electron microscope EM-900 (Zeiss, Germany).

2.3. Immunohistochemistry for uPAR. Renal biopsy sections were fixed in paraformaldehyde for immunohistochemistry, and peroxidase and protein blockage was done using Novolink blocker for 50 minutes each. Then, human anti-*uPAR* antibody (1 : 50) was incubated overnight at 4°C. After, slides were incubated with Post Primary (Novolink Polymer Detection System Kit, BL, UK) for 50 minutes at room temperature and then incubated with the polymer (Novolink Polymer Detection System Kit, BL, UK) for 50 minutes. The material was then allowed to react with DAB substrate for staining (1,4-dideoxy-1,4-imino-D-arabinitol-diaminobenzidine) (Liquid DAB, Dako, Carpinteria, CA, USA) for 2 minutes, and sections were counterstained with hematoxylin and analyzed using a light microscope.

2.4. uPAR Immunostaining Quantification. Immunostained cells in glomeruli were counted as uPAR-positive cells, in order to obtain its density in a glomerular area. The result was expressed in cell density (cell/mm^2), in a technique adapted from Venkatareddy et al. [27].

2.5. Statistical Analysis. Statistical analysis was performed with the program GraphPad Prism version 6.0. Normality was tested by the Shapiro-Wilk test. For comparison analysis, the Kruskal-Wallis test (H) was used followed by the Dunn posttest. In contingency table analysis, Fisher's exact test was used. uPAR diagnostic performance in renal biopsy was evaluated with the receiver operating characteristic curve (ROC curve) using sensitivity, specificity, and area under the curve (AUC) with 95% of confidence intervals (CI); cutoff points were calculated using nonparametric methods. Differences were considered statistically significant when $p < 0.05$.

3. Results

Patients' median age was 35.5 years, ranging from 15 to 70 years. Twenty were men and 18 were women. Patients with FSGS presented a more unfavorable clinical profile, with higher levels of creatinine ($p = 0.0156$; $U = 56.50$), of proteinuria ($p = 0.0234$; $U = 102.0$), and increased prevalence of hypertension (Fisher's exact test $p = 0.0009$). Patients' profile is detailed in Table 1.

A previous study has shown that suPAR may be related to FSGS pathogenesis. So, we hypothesized that patients with FSGS have greater in situ uPAR expression in glomeruli than patients with MCD. It was observed that uPAR podocyte expression was increased in the FSGS group (Figure 1(c)) compared to both the control group (Figure 1(a); $p = 0.0076$; Figure 1(d)) and MCD group (Figure 1(b); $p = 0.0368$; Figure 1(d)). The expression of uPAR was diffuse in glomeruli without sclerosis and in viable glomerulus cells within segmental sclerosis.

As uPAR expression was increased in biopsies of patients with FSGS, we sought to examine how useful uPAR immunohistochemistry staining would be to FSGS diagnosis. Using a ROC curve, an optimum cutoff point at 0.08 cells/mm^2 labeled with uPAR was found to have 64.29% of sensitivity, 80.95% of specificity, and AUC of 0.7670 (95% CI of 0.5870-0.9470, $p = 0.008$, Figure 2(b)). The same was not observed when the ROC curve was used to evaluate potential diagnosis of uPAR in MCD ($p = 0.3937$, Figure 2(a)).

Table 1: Clinical-epidemiological profile of patients.

	MCD ($n = 16$)	FSGS ($n = 22$)	p value
Age			
Mean ± SD	37.37 ± 14.90	38.32 ± 14.92	0.8484
Median (min–max)	34.5 (15-70)	36 (16-73)	
Gender, n (%)			
Male	6 (37.5%)	14 (63.64%)	0.1881
Female	10 (62.5%)	8 (36.36%)	
Creatinine (mg/dl)			
Mean ± SD	0.97 ± 0.51	1.52 ± 0.65	0.0156*
Median (min–max)	0.9 (0.5-2.4)	1.4 (0.8-3.0)	
Proteinuria (g/24 h)			
Mean ± SD	2.83 ± 2.15	5.13 ± 3.37	0.0234*
Median (min–max)	2.15 (0.16-6.19)	4.35 (1.24-14.46)	
Albumin (mg/dl)			
Mean ± SD	2.85 ± 1.01	2.72 ± 1.01	0.8258
Median (min–max)	2.6 (1.4-4.6)	3.3 (0.8-3.9)	
Hematuria			
Yes	8 (50.00%)	6 (27.27%)	0.4905
No	8 (50.00%)	11 (50.00%)	
Hypertension			
Yes	4 (25.00%)	16 (72.73%)	0.0009*
No	8 (50.00%)	1 (4.54%)	

*$p < 0.05$.

4. Discussion

FSGS and MCD are common glomerular diseases which present clinical similarities as proteinuria and/or nephrotic syndrome but have different clinical evolution. In this study, patients with FSGS presented higher serum levels of creatinine and presence of arterial hypertension, which is consistent with literature, as this entity presents an unfavorable clinical course, does not respond well to corticosteroids, and progresses to renal failure in a variable period of time [28]. About 25 to 50% of patients with FSGS have decreased renal function, and arterial hypertension is present in about 60% of them [29].

In addition to clinical similarities, both entities present morphological similarities as foot process effacement, and, in cases in which FSGS sclerosis is not sampled, the differential diagnosis between these two diseases becomes challenging.

In this scenario, we looked for a possible biomarker that would help differentiate these two entities. We chose uPAR/suPAR, which has been proposed to have a role in FSGS pathogenesis [11–15].

Of note, our MDC patients had low levels of proteinuria, which is not in line with literature [29]. However, these patients presented important hypoalbuminemia, characterizing the nephrotic condition. This hypoalbuminemia reflects low levels of serum protein, which results in less protein in urine. Although there is a consensus recommendation for renal biopsy only in patients with nephrotic range proteinuria, renal biopsy indications differ considerably among nephrologists [30]. Thus, it is possible that the presence of hypoalbuminemia in our patients contributed to the indication of biopsy even with nonnephrotic proteinuria.

In this study, we demonstrate that patients with FSGS have increased uPAR expression in renal biopsy compared to patients without renal alteration and patients with MCD.

The protein uPAR is expressed in human glomerular cells and one of them is the podocyte, as verified by double immunofluorescence labeling with synaptopodin, a podocyte marker. The same was observed in glomeruli of animal models, where uPAR expression in all models of proteinuria was substantially increased in glomerular cells, including podocytes. By analyzing vitronectin expression, a protein that binds to uPAR, a labeling pattern like that of uPAR was observed in human and animal podocytes. In addition, culture of podocytes treated with puromycin aminonucleoside (PAN) and LPS revealed increased uPAR expression in these cells with labeling preferentially located on cell membrane [18].

Using an animal model knockout for PLAUR, uPAR has been shown to play a direct role in the regulation of podocyte structure and function, as uPAR deficiency protects against LPS-induced proteinuria and podocyte injury [18].

A possible mechanism for foot process effacement is through integrin activation as uPAR is a GPI-anchored protein without a cytoplasmic tail and uPAR signal

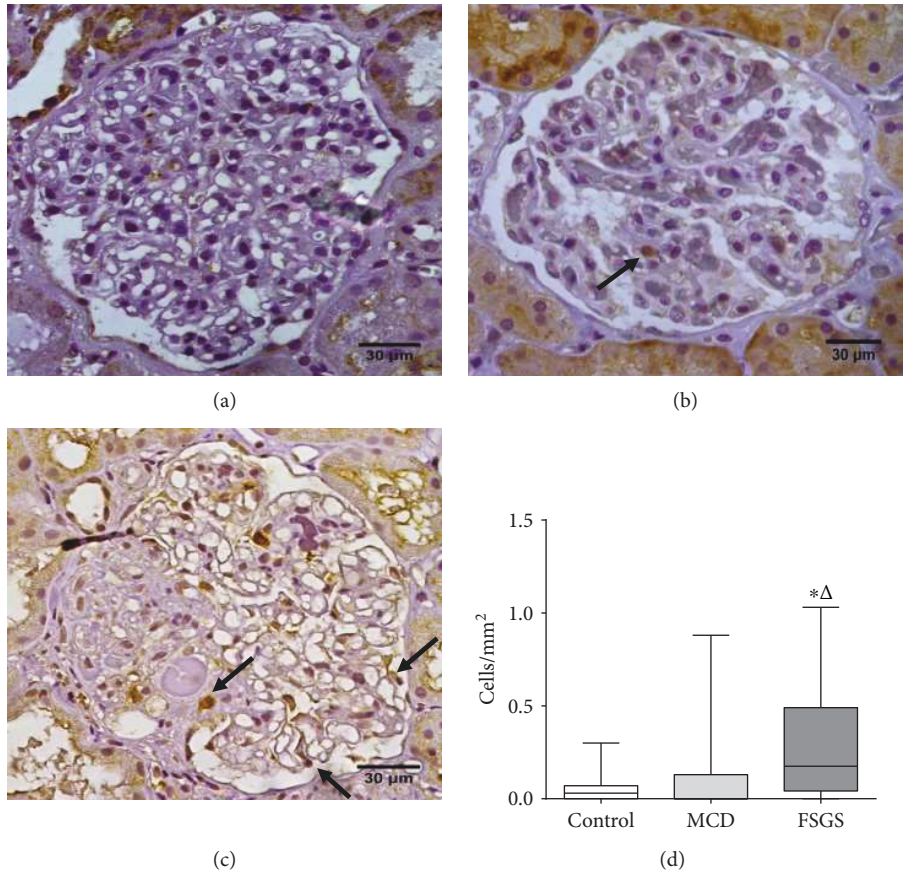

Figure 1: Evaluation of uPAR in podocytopathies and in the control group. uPAR immunolabeling evidenced by arrows (a) in the control group; (b) in a case of MCD, in which only rare cells are labeled; and (c) in a case of FSGS in which there are diffusely marked cells, including cells still viable in the sclerotic segment. (d) uPAR glomerular expression. Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparison. Horizontal lines represent the median, bars represent 25-75% percentiles, and vertical lines represent 10-90% percentiles. MCD: minimal change disease; FSGS: focal segmental glomerulosclerosis; ∗: significant differences between the FSGS versus the control group; Δ: significant differences between the FSGS versus the MCD group.

transduction seems to be through lateral interactions with membrane proteins such as integrins [31].

After identification of an integrin-interacting sequence located in domain 2 of uPAR that activates the $\alpha v\beta 3$ signal-dependent signaling pathways [32], it was observed through immunogold that the location of $\alpha v\beta 3$ integrin and uPAR was similar in podocytes, suggesting uPAR interacts with this integrin. In addition, using an antibody that inhibits $\beta 3$ integrin function, mice did not develop proteinuria in response to LPS [31]. Another evidence of uPAR interaction with $\alpha v\beta 3$ integrin comes from an experiment with animal knockout for urokinase, the major ligand of uPAR, in which, after treatment with LPS, animals presented proteinuria, showing podocyte lesion triggered by uPAR is independent of its ligand [18].

It is believed that both urinary and serum suPAR can activate $\beta 3$ integrin in a similar manner to the binding of uPAR to podocyte membrane. To study human podocytes $\beta 3$ integrin activity, AP5, an epitope-recognizing antibody was used and a strong AP5 labeling was observed along cell membrane of podocytes incubated with urine of FSGS patients. This expression was reduced with the addition, in the incubation, of an antibody blocking uPAR [26]. The same was observed in podocytes cultured with plasma of patients with recurrent FSGS [11].

The role of uPAR/suPAR as a FSGS biomarker is still controversial in literature. However, in this study involving podocytopathies, we observed that uPAR has a specificity for FSGS and can be considered a scaring factor in this disease.

4.1. Limitation of Study. Although our sample came from several Brazilian regions, it would be advisable that studies involving human samples would be replicated in different cohorts and ethnicities.

5. Conclusions

Our results demonstrated that uPAR has high specificity for FSGS cases. Therefore, this marker may be useful in the diagnosis of FSGS in renal biopsies in which FSGS is suspected, but the sclerotic lesion was not sampled.

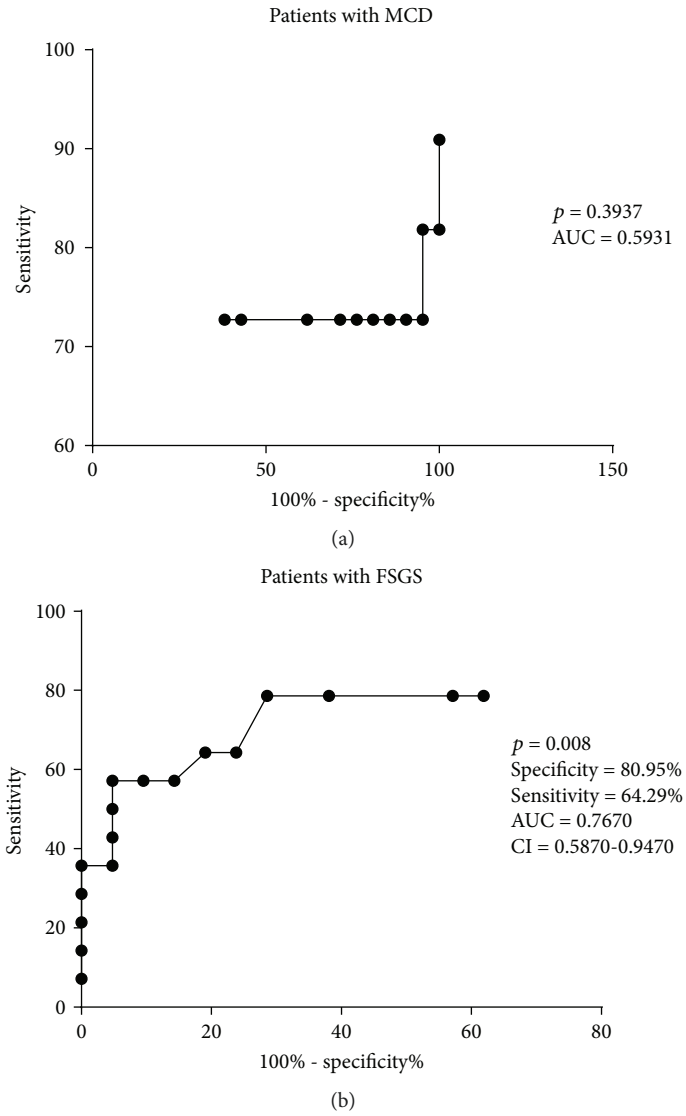

Figure 2: Receiver operating characteristic (ROC) curve to evaluate the potential diagnosis of uPAR. (a) ROC curve showed that uPAR has no potential diagnosis in patients with MCD. (b) ROC curve showed that uPAR can be considered a potential biomarker for FSGS. AUC: area under the curve; CI: confidence interval.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

There is no conflict of interest.

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References

[1] A. Sinha and A. Bagga, "Nephrotic syndrome," *The Indian Journal of Pediatrics*, vol. 79, no. 8, pp. 1045–1055, 2012.

[2] T. A. Wynn, "Cellular and molecular mechanisms of fibrosis," *The Journal of Pathology*, vol. 214, no. 2, pp. 199–210, 2008.

[3] R. Lennon, M. J. Randles, and M. J. Humphries, "The importance of podocyte adhesion for a healthy glomerulus," *Frontiers in Endocrinology*, vol. 5, p. 160, 2014.

[4] H. Cheng and R. C. Harris, "The glomerulus – a view from the outside – the podocyte," *The International Journal of Biochemistry & Cell Biology*, vol. 42, no. 9, pp. 1380–1387, 2010.

[5] A. K. Büscher and S. Weber, "Educational paper: the podocytopathies," *European Journal of Pediatrics*, vol. 171, no. 8, pp. 1151–1160, 2012.

[6] C. Aparecida da Silva, M. Molinar Mauad Cintra, E. de Castro Côbo et al., "Renal biopsy: use of biomarkers as a tool for the diagnosis of focal segmental glomerulosclerosis," *Disease Markers*, vol. 2014, Article ID 192836, 11 pages, 2014.

[7] J. R. Machado, L. P. Rocha, P. D. M. d. M. Neves et al., "An overview of molecular mechanism of nephrotic syndrome," *International Journal of Nephrology*, vol. 2012, Article ID 937623, 6 pages, 2012.

[8] A. Z. Rosenberg and J. B. Kopp, "Focal segmental glomerulosclerosis," *Clinical Journal of the American Society of Nephrology*, vol. 12, no. 3, pp. 502–517, 2017.

[9] M. Vivarelli, L. Massella, B. Ruggiero, and F. Emma, "Minimal change disease," *Clinical Journal of the American Society of Nephrology*, vol. 12, no. 2, pp. 332–345, 2017.

[10] R. J. Maas, J. K. Deegens, B. Smeets, M. J. Moeller, and J. F. Wetzels, "Minimal change disease and idiopathic FSGS: manifestations of the same disease," *Nature Reviews Nephrology*, vol. 12, no. 12, pp. 768–776, 2016.

[11] C. Wei, S. el Hindi, J. Li et al., "Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis," *Nature Medicine*, vol. 17, no. 8, pp. 952–960, 2011.

[12] C. Wei, H. Trachtman, J. Li et al., "Circulating suPAR in two cohorts of primary FSGS," *Journal of the American Society of Nephrology*, vol. 23, no. 12, pp. 2051–2059, 2012.

[13] J. Huang, G. Liu, Y. M. Zhang et al., "Plasma soluble urokinase receptor levels are increased but do not distinguish primary from secondary focal segmental glomerulosclerosis," *Kidney International*, vol. 84, no. 2, pp. 366–372, 2013.

[14] G. Cara-Fuentes, C. Wei, A. Segarra et al., "CD80 and suPAR in patients with minimal change disease and focal segmental glomerulosclerosis: diagnostic and pathogenic significance," *Pediatric Nephrology*, vol. 29, no. 8, pp. 1363–1371, 2014.

[15] F. Li, C. Zheng, Y. Zhong et al., "Relationship between serum soluble urokinase plasminogen activator receptor level and steroid responsiveness in FSGS," *Clinical Journal of the American Society of Nephrology*, vol. 9, no. 11, pp. 1903–1911, 2014.

[16] M. Thunø, B. Macho, and J. Eugen-Olsen, "suPAR: the molecular crystal ball," *Disease Markers*, vol. 27, no. 3-4, pp. 157–172, 2009.

[17] H. W. Smith and C. J. Marshall, "Regulation of cell signalling by uPAR," *Nature Reviews Molecular Cell Biology*, vol. 11, no. 1, pp. 23–36, 2010.

[18] C. Wei, C. C. Möller, M. M. Altintas et al., "Modification of kidney barrier function by the urokinase receptor," *Nature Medicine*, vol. 14, no. 1, pp. 55–63, 2008.

[19] J. Reiser, C. C. Nast, and N. Alachkar, "Permeability factors in focal and segmental glomerulosclerosis," *Advances in Chronic Kidney Disease*, vol. 21, no. 5, pp. 417–421, 2014.

[20] B. Meijers, R. J. H. Maas, B. Sprangers et al., "The soluble urokinase receptor is not a clinical marker for focal segmental glomerulosclerosis," *Kidney International*, vol. 85, no. 3, pp. 636–640, 2014.

[21] A. Segarra, E. Jatem, M. T. Quiles et al., "Diagnostic value of soluble urokinase-type plasminogen activator receptor serum levels in adults with idiopathic nephrotic syndrome," *Nefrología*, vol. 34, no. 1, pp. 46–52, 2014.

[22] T. Wada, M. Nangaku, S. Maruyama et al., "A multicenter cross-sectional study of circulating soluble urokinase receptor in Japanese patients with glomerular disease," *Kidney International*, vol. 85, no. 3, pp. 641–648, 2014.

[23] J. M. Spinale, L. H. Mariani, S. Kapoor et al., "A reassessment of soluble urokinase-type plasminogen activator receptor in glomerular disease," *Kidney International*, vol. 87, no. 3, pp. 564–574, 2015.

[24] J. Reiser, C. Wei, and J. Tumlin, "Soluble urokinase receptor and focal segmental glomerulosclerosis," *Current Opinion in Nephrology and Hypertension*, vol. 21, no. 4, pp. 428–432, 2012.

[25] J. Reiser, "Circulating permeability factor suPAR: from concept to discovery to clinic," *Transactions of the American Clinical and Climatological Association*, vol. 124, pp. 133–138, 2013.

[26] J. Huang, G. Liu, Y. M. Zhang et al., "Urinary soluble urokinase receptor levels are elevated and pathogenic in patients with primary focal segmental glomerulosclerosis," *BMC Medicine*, vol. 12, no. 1, p. 81, 2014.

[27] M. Venkatareddy, S. Wang, Y. Yang et al., "Estimating podocyte number and density using a single histologic section," *Journal of the American Society of Nephrology*, vol. 25, no. 5, pp. 1118–1129, 2014.

[28] P. Cravedi, J. B. Kopp, and G. Remuzzi, "Recent progress in the pathophysiology and treatment of FSGS recurrence," *American Journal of Transplantation*, vol. 13, no. 2, pp. 266–274, 2013.

[29] J. A. Jefferson, P. J. Nelson, B. Najafian, and S. J. Shankland, "Podocyte disorders: Core Curriculum 2011," *American Journal of Kidney Diseases*, vol. 58, no. 4, pp. 666–677, 2011.

[30] G. Fuiano, G. Mazza, N. Comi et al., "Current indications for renal biopsy: a questionnaire-based survey," *American Journal of Kidney Diseases*, vol. 35, no. 3, pp. 448–457, 2000.

[31] F. Blasi and P. Carmeliet, "uPAR: a versatile signalling orchestrator," *Nature Reviews Molecular Cell Biology*, vol. 3, no. 12, pp. 932–943, 2002.

[32] B. Degryse, M. Resnati, R. P. Czekay, D. J. Loskutoff, and F. Blasi, "Domain 2 of the urokinase receptor contains an integrin-interacting epitope with intrinsic signaling activity: generation of a new integrin inhibitor," *Journal of Biological Chemistry*, vol. 280, no. 26, pp. 24792–24803, 2005.

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Research Article

Urinary TIMP2 and IGFBP7 Identifies High Risk Patients of Short-Term Progression from Mild and Moderate to Severe Acute Kidney Injury during Septic Shock: A Prospective Cohort Study

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Background. To examine whether the new urinary biomarkers TIMP2 and IGFBP7 can predict progression within 24 hours and 72 hours from mild and moderate (KDIGO 1 or 2) to severe (KDIGO 3) AKI in patients with septic shock. *Methods*. A prospective, multicenter observational study performed in three French ICUs. The urinary biomarkers TIMP2∗IGFBP7 were analyzed at the early phase (<6 hours) of patients admitted for septic shock with mild and moderate AKI. *Results*. Among the 112 patients included, 45 (40%) progressed to the KDIGO 3 level 24 hours after inclusion (KDIGO 3 H24) and 47 (42%) 72 hours after inclusion (KDIGO 3 H72). The median urinary TIMP2∗IGFBP7 at inclusion (baseline) were higher in the KDIGO 3 group than in the KDIGO<3 group at H24 and H72. All covariates with a p value < 0.1 in the univariate analysis were included in stepwise multiple logistic regression models to identify factors independently associated with the risk of KDIGO 3 at H24 and H72. TIMP2∗IGFBP7 remained independently associated with KDIGO 3 at H24 and H72. Baseline posology of norepinephrine, baseline urine output, and baseline serum creatinine remained also significantly associated with progression to KDIGO 3 at H24. Baseline TIMP2∗IGFBP7 and baseline urinary output had the best AUC ROC. A baseline TIMP2∗IGFBP7 > 2.0 $(ng/ml)^2/1{,}000$ identified the population at high risk of KDIGO 3 H24 (relative risk 4.19 (1.7-10.4)) with a sensitivity of 76% (60-87) and a specificity of 81% (69-89). But the diagnostic performance at H72 of baseline TIMP2∗IGFBP7 was poor (AUC: 0.69 (0.59-0.77)). *Conclusion*. The urinary TIMP2∗IGFBP7 concentration and the urine output at the early phase of septic shock are independent factors to identify the population at high risk of progression from mild and moderate to severe AKI over the next 24 but not 72 hours. A TIMP2∗IGFBP7 concentration > 2.0 $(ng/ml)^2/1{,}000$ quadruples the risk of KDIGO 3 AKI within 24 hours. This trial is registered with (NCT03547414).

1. Background

Septic shock is one of the leading causes of death in patients admitted to the intensive care unit (ICU) [1]. Acute kidney injury (AKI) occurs in almost 50% of septic patients and is associated with significant mortality [2]. The definition and staging of AKI have been standardized. The Kidney Disease: Improving Global Outcomes (KDIGO) consensus classification defines three stages of AKI (AKIN classification) based on the patient's urine output and serum creatinine [3]. The survival rate of ICU patients decreases with incremental staging of this classification [4]. Patients who will progress to severe AKI (KDIGO stage 3) and who present the highest risk of death can be poorly discriminated from patients who will remain below stage 3 and eventually return to normal kidney function (transient AKI) [5]. Renal biomarkers are unable to accurately identify those patients who will progress to severe AKI (KDIGO 3) [5].

A new urine test, the NephroCheck™, has been validated [6]. It corresponds to the product of the urinary concentrations of 2 markers of renal tubule injury (TIMP2 and IGFBP7) associated with a risk of developing AKI KDIGO 2 or 3 within 12 hours. The product of the urinary concentrations of TIMP2 and IGFBP7, two cell cycle arrest proteins, has been shown to predict the development of AKI within 12 to 24 hours [7, 8]. The studies have been performed in unselected critical care patients [9] and in different settings: emergency room [10], postcardiac surgery [11], and septic patients [12]. TIMP2 and IGFBP7 are both released by tubular cells exposed to septic and/or haemodynamic aggression. Although TIMP2∗IGFBP7 can distinguish patients who will subsequently develop AKI, it is unknown whether the baseline urinary concentrations of these biomarkers are also associated with the risk of progression from mild and moderate (KDIGO 1 or 2) to severe (KDIGO 3) AKI. The aim of this study was to determine whether the urinary TIMP2∗IGFBP7 concentration can identify patients with a high risk of progression to KDIGO level 3 in patients with septic shock and AKI KDIGO 1 or 2.

2. Methods

This protocol was approved by the Institutional Review Board (IRB North-West II). Patients or their surrogates were informed and could decline to participate at any time, and their decision was recorded in the patient's files.

All patients admitted in the 16-bed medical ICU in Amiens University Hospital (France) between March 2014 and September 2016 and all patients admitted in the 32-bed medical ICU in Montpellier University Hospital (France) and the 22-bed medical and surgical ICU in Melun General Hospital (France) between September 2015 and September 2016 with septic shock according to the bone criteria and AKI KDIGO 1 or 2 within 6 hours after initiation of catecholamines were prospectively included in this study in order to determine the diagnostic value of TIMP2∗IGFBP7 to identify patients at high risk of KDIGO 3. Exclusion criteria were patients without AKI (KDIGO 0), anuria, severe AKI (KDIGO 3), chronic kidney disease (creatinine clearance < 15 ml/min), decision to withhold treatment, cardiac arrest, age < 18 years, or pregnancy.

Basal serum creatinine was defined as the serum creatinine level measured during the 12 months preceding the onset of septic shock. In the absence of a previous serum creatinine assay, basal serum creatinine was estimated according to the KDIGO guideline [3]. All "baseline" parameters have been recorded at inclusion. Baseline urine output was the volume of urine excreted by hour during the 4 to 6 hours preceding the inclusion.

2.1. Measurements. A fresh urine sample was collected on inclusion (maximum 6 hours after starting catecholamines) through the urine collecting tube and frozen at -80°C. At the end of the study, urine samples were thawed and centrifuged as recommended by the manufacturer and the urinary TIMP2∗IGFBP7 concentration was determined using the NephroCheck™ test (Astute Medical Inc., San Diego, CA, USA). The NephroCheck™ test simultaneously measures into the Astute 140™ Meter (a benchtop analyzer) the urinary concentrations of TIMP2 and IGFBP7 on 100 μl of urine mixed with 100 μl of buffer. The result is expressed as a single number corresponding to the product of the TIMP2 and IGFBP7 concentrations. The coefficients of variation (CV) given by the manufacturer for the interassay are comprised of between 8.1% and 11.4% for TIMP2 and between 6.6% and 7.9% for IGFBP7. The CV for the intra-assay are comprised of between 8.0% and 10.7% for TIMP2 and 6.3% and 7.7% for IGFBP7.

2.2. Study Endpoints. AKI was categorized according to KDIGO guidelines at baseline, 24 hours, and 72 hours after inclusion [3].

Patients were classified 24 and 72 hours after inclusion according to the progression of the KDIGO classification or death: patients who remained KDIGO<3 and patients who progressed to KDIGO 3 or died.

2.3. Statistics. Results are expressed as median (95% confidence interval), and categorical variables are expressed as n (%). Comparisons between groups were performed using the Mann-Whitney test or chi-square test, as appropriate.

The performance of the different parameters to identify patients with high risk of deterioration of AKI to KDIGO 3 was tested using multiple logistic regression models including all covariates with a p value < 0.1 in the univariate analysis. All significant variables in the first logistic regression model were than retested in a second logistic regression model. Because the diagnostic performance of the various parameters depends on the predictive time frame, analyses were repeated to predict KDIGO 3 at 24 hours and KDIGO 3 or death at 72 hours after inclusion. Nagelkerke pseudo-R^2, a marker of the strength of the final model, is presented. A Nagelkerke pseudo-R^2 close to 1 indicates that the full model reliably predicts the outcome.

The diagnostic value of each parameter independently associated with the progression to KDIGO 3 in the logistic regression models was determined using receiver operating characteristic (ROC) curve analysis. The best parameter was determined by comparing the area under the curve (AUC) using the Hanley-McNeil test at each time point. The best cutoff values to identify patients at high risk of progression to KDIGO 3 at H24 and H72 were determined. Also, we looked at the existing cutoffs for TIMP2∗IGFBP7 (0.3 and 2.0 $(\text{ng/ml})^2/1{,}000$) described previously [9]. Sensitivity, specificity, and negative and positive predictive values were calculated. A p value < 0.05 was considered significant. Statistical analysis was performed using MedCalc version 18.6 (MedCalc Software, Mariakerke, Belgium) software.

3. Results

3.1. Study Endpoints and Population Characteristics. During the study period, 2,800 patients were admitted in the 3 participating centers, including 825 patients with septic shock. One hundred and twelve of these patients were included in the study, while 713 patients were excluded because they

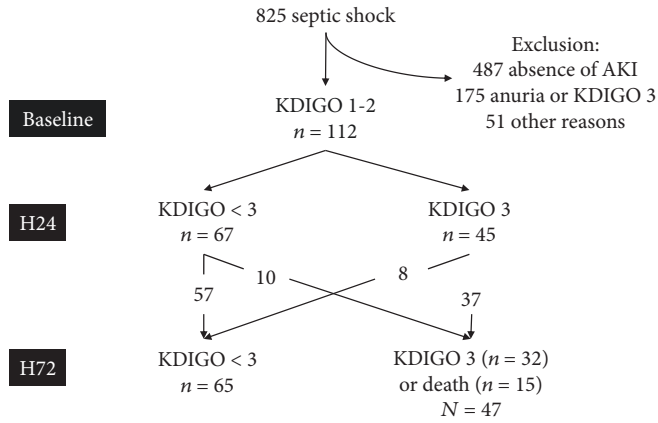


Figure 1: Flow chart.

presented at least one exclusion criterion: 487 because of the absence of AKI, 175 because of severe AKI (anuria or KDIGO 3 at admission), and the remaining 51 for various reasons (cardiac arrest, chronic renal failure, or withholding of treatment).

During the 24 hours following inclusion, 45 patients developed KDIGO 3 AKI (diagnostics based on creatinine elevation in 14 patients, low urinary output in 9 patients, and both criteria in 22 patients) and 67 remained below KDIGO 3. At H72, 32 patients were KDIGO 3, 65 were KDIGO<3, and 15 patients had died (Figure 1).

3.2. Identification of Patients at High Risk of Severe AKI at H24. Patients with a diagnosis of KDIGO 3 AKI 24 hours following inclusion (KDIGO 3 H24) presented at inclusion a higher posology of norepinephrine, volume of fluid administered, and lactate level (Table 1). The rate of mortality in the KDIGO 3 group of patients was higher than that in the KDIGO<3 patients (28 (62%) vs. 21 (31%), respectively; $p = 0.002$). The KDIGO 3 H24 group had a significantly higher median baseline urinary TIMP2∗IGFBP7 concentration (3.99 (2.17-9.45) $(\text{ng/ml})^2/1{,}000$) compared to the KDIGO<3 H24 group (0.93 (0.25-1.59); $p = 0.001$) (Table 1 and Figure 2). Median baseline urine output and baseline serum creatinine were, respectively, higher and lower in the KDIGO<3 H24 group than in the KDIGO 3 H24 group (Table 1).

In the logistic regression model, only the baseline posology of norepinephrine, baseline TIMP2∗IGFBP7, baseline urine output, and baseline serum creatinine remained significantly associated with progression to KDIGO 3 at H24 (Table 2).

The value of urinary TIMP2∗IGFBP7 concentration to identify patients at high risk of KDIGO stage 3 during the 24 hours following inclusion was superior to that of the baseline serum creatinine (AUC: 0.83 (0.75-0.90) vs. 0.70 (0.61-0.79), respectively; $p = 0.04$) and baseline posology of norepinephrine (0.69 (0.60-0.78), $p = 0.03$) but was not significantly different to that of the baseline urine output (0.73 (0.63-0.81); $p = 0.08$) (Figure 3). A TIMP2∗IGFBP7 > 1.92 $(\text{ng/ml})^2/1{,}000$ identified patients who progressed to the KDIGO 3 within 24 hours with a sensitivity of 78% (63-89), a specificity of 81% (69-89), a positive predictive value of 73% (58-84), and a negative predictive value of 84% (73-92). The TIMP2∗IGFBP7 AUC to identify KDIGO 3 were not significantly different between the KDIGO 1 ($n = 73$) and KDIGO 2 ($n = 39$) patients at admission ($p = 0.09$).

The previously published cutoffs for TIMP2∗IGFBP7 to identify critically ill patients at high risk of developing moderate to severe AKI were 0.3 and 2.0 $(\text{ng/ml})^2/1{,}000$ defining the lowest risk to develop an AKI as below 0.3, the intermediate risk as between 0.3 and 2.0, and high risk if higher than 2.0 (9). In our population, a TIMP2∗IGFBP7 < 0.3 identified the group of patients with the lowest risk of developing KDIGO 3 with a sensitivity of 30% (19-42) and specificity of 91% (79-97). A TIMP2*IGFBP7 > 2.0 identified the population at high risk of KDIGO 3 with a sensitivity of 76% (60-87) and a specificity of 81% (69-89). Compared with patients with a TIMP2∗IGFBP7 below 0.3, those with a test score between 0.3 and 2.0 had the same risk for severe AKI (relative risk 0.84 (0.3-2.7, $p = 0.77$)) whereas those with a test score > 2.0 had 4 times the risk for severe AKI (4.19 (1.7-10.4, $p = 0.002$)) (Figure 4).

3.3. Identification of Patients at High Risk of Severe AKI at H72. Patients with a diagnosis of KDIGO 3 72 hours following inclusion (KDIGO 3 H72) had a higher posology of norepinephrine, volume of fluid administered, lactate level, and lower mean arterial pressure at inclusion (Table 1). Urine output (0.71 (0.54-0.95) vs. 0.31 (0.2-0.49) ml/kg/h; $p = 0.001$), serum creatinine (114 (106-131) vs. 139 (122-170) μmol/l; $p = 0.009$), and TIMP2∗IGFBP7 (1.03 (0.77-1.51) vs. 3.03 (1.81-5.11); $p = 0.001$) at inclusion were significantly different between the KDIGO<3 H72 and KDIGO 3 H72 groups (Table 1 and Figure 2). In the logistic regression model including the presence of baseline mean arterial pressure, baseline posology of norepinephrine, baseline fluid administered, baseline lactate, baseline TIMP2∗IGFBP7, baseline urine output, and baseline serum creatinine, only the baseline creatinine and baseline TIMP2∗IGFBP7 remained significantly associated with progression to

Table 1: Clinical characteristics and comparison between patients who did not develop severe AKI (KDIGO<3) and those who developed severe AKI (KDIGO 3) during the 24 hours and 72 hours following inclusion.

Parameter	Global $n = 112$	KDIGO<3 H24 $n = 67$	KDIGO 3 H24 $n = 45$	p value	KDIGO<3 H72 $n = 65$	KDIGO 3 or death H72 $n = 47$	p value
Age (y)	65 (59-75)	65 (55-76)	64 (60-74)	0.93	65 (62-70)	65 (62-71)	0.85
Male (n (%))	67 (60)	41 (61)	26 (58)	0.87	40 (61)	27 (57)	0.70
SAPS II	57 (43-70)	55 (42-64)	64 (51-76)	0.01	52 (47-57)	67 (61-73)	0.001
BMI (kg/m^2)	27 (24-32)	28 (24-33)	26 (22-30)	0.21	28 (25-33)	26 (23-33)	0.56
Basal creatinine (μmol/l)	71 (60-92)	76 (61-94)	71 (57-87)	0.37	73 (69-80)	71 (66-79)	0.44
Comorbidity (n (%))							
Diabetes	46 (41)	30 (45)	16 (35)	0.43	29 (45)	17 (36)	0.44
Hypertension	56 (50)	34 (51)	22 (49)	0.99	33 (51)	23 (49)	0.99
Cardiovascular disease	22 (20)	14 (21)	8 (18)	0.49	13 (20)	9 (19)	0.99
Any malignancy	34 (30)	17 (25)	17 (38)	0.23	16 (25)	18 (38)	0.15
Origin of sepsis (n (%))							
Pulmonary	73 (65)	43 (64)	30 (67)	0.88	43 (64)	30 (64)	0.9
Gastrointestinal	13 (12)	8 (12)	5 (11)	1	7 (11)	6 (13)	0.91
Soft tissue	7 (6)	4 (6)	3 (7)	0.9	5 (6)	2 (4)	0.73
Urinary	7 (6)	6 (9)	1 (2)	0.4	6 (9)	1 (2)	0.25
Other	5 (4)	3 (4)	2 (4)	1	4 (4)	1 (2)	0.82
Baseline MAP (mmHg)	73 (66-86)	74 (67-87)	71 (62-82)	0.15	74 (70-82)	71 (66-76)	0.02
Baseline heart rate (bpm)	105 (90-123)	101 (87-121)	110 (93-127)	0.17	104 (97-109)	111 (100-114)	0.20
Baseline norepinephrine (μg/kg/min)	0.36 (0.16-0.87)	0.27 (0.15-0.45)	0.60 (0.30-1.16)	0.001	0.32 (0.20-0.37)	0.50 (0.29-0.87)	0.03
Fluid administered (between initiation of catecholamines and first urine sample) (ml/kg)	16 (7-25)	15 (5-23)	19 (11-28)	0.01	15 (9-17)	20 (14-25)	0.01
Baseline lactate (mmol/l)	2.6 (1.6-4.2)	2.2 (1.6-3.7)	3.0 (2.0-5.4)	0.03	2.1 (1.8-2.5)	3.5 (2.9-4.4)	0.001
Mechanical ventilation (n (%))	77 (69)	46 (69)	31 (69)	0.85	42 (65)	35 (74)	0.31
Interval between initiation of catecholamines and first urine sample (hours)	2.0 (1.0-4.0)	2.0 (1.0-4.0)	3.0 (1.0-4.0)	0.24	2.0 (2.0-2.7)	3.0 (2.0-3.0)	0.46
Mortality in ICU (n (%))	49 (44)	21 (31)	28 (62)	0.002	18 (28)	31 (66)	0.001
ICU length of stay (days)	6.0 (3-14)	7.0 (4.0-15.7)	5.0 (2.5-12.0)	0.05	8.0 (6.0-10.9)	4.0 (3.0-8.1)	0.09
Renal replacement therapy within 72 hours (n (%))	19 (17)	3 (4)	16 (35)	0.001	2 (3)	17 (36)	0.001
Baseline urine output (ml/kg/h)	0.53 (0.24-1.19)	0.73 (0.40-1.47)	0.31 (0.16-0.64)	0.001	0.71 (0.54-0.95)	0.31 (0.20-0.49)	0.001
Baseline creatinine (μmol/l)	123 (91-174)	114 (87-139)	163 (108-247)	0.001	114 (106-131)	139 (122-170)	0.009
Baseline TIMP2∗IGFBP7 ((ng/ml)^2/1,000)	1.45 (0.58-4.13)	0.93 (0.25-1.59)	3.99 (2.17-9.45)	0.001	1.03 (0.77-1.51)	3.03 (1.81-5.11)	0.001

SAPS II: Simplified Acute Physiology Score 2; MAP: mean arterial pressure; ICU: intensive care unit; H0: time of first urine sample. Basal serum creatinine was defined as the serum creatinine level measured during the 12 months preceding the onset of septic shock. In the absence of a previous serum creatinine assay, basal serum creatinine was estimated according to the KDIGO guideline [3]. All baseline parameters have been recorded at inclusion. Baseline urine output was the volume of urine excreted by hour during the 4 to 6 hours preceding the inclusion.

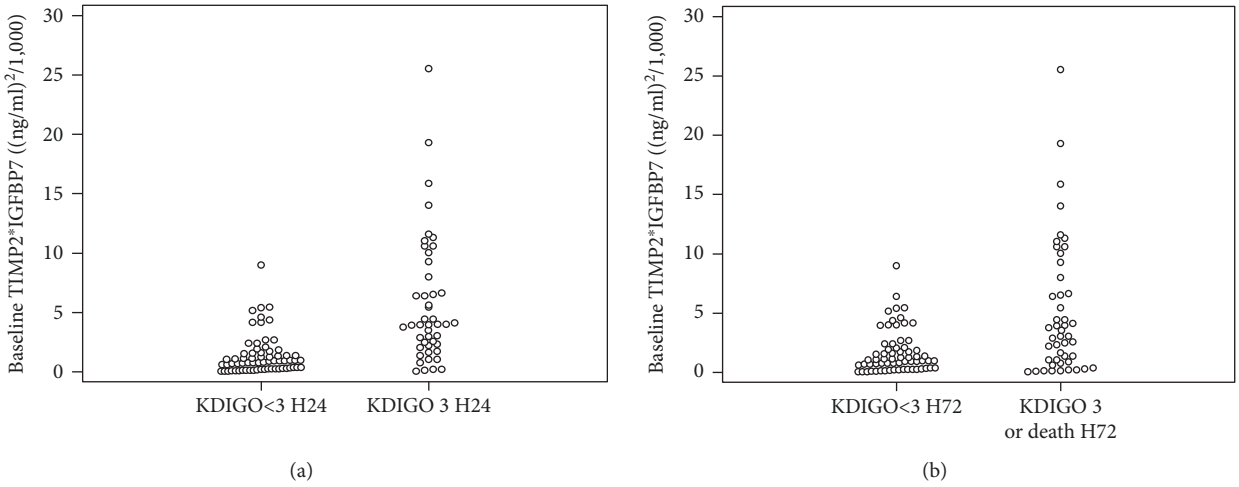


Figure 2: Individual baseline TIMP2∗IGFBP7 in patients who developed and who did not develop KDIGO 3 AKI at H24 (a) and H72 (b).

Table 2: Stepwise logistic regression risk models for severe AKI 24 hours (H24) or severe AKI and/or death 72 hours (H72) following inclusion.

	AKI KDIGO H24			
Variable	H24 (OR (95% CI))	p value	H24 (OR (95% CI))	p value
Baseline posology of norepinephrine	4.26 (1.16-15.6)	0.03	4.55 (1.43-14.5)	0.01
Baseline fluid administered	1.02 (0.97-1.06)	0.47	—	—
Baseline lactate	1.02 (0.80-1.29)	0.87	—	—
Baseline creatinine	1.01 (1.01-1.02)	0.001	1.02 (1.01-1.3)	0.001
Baseline urine output	0.19 (0.05-0.77)	0.02	0.19 (0.05-0.74)	0.004
Baseline TIMP2∗IGFBP7	1.42 (1.11-1.83)	0.005	1.42 (1.12-1.81)	0.001
Nagelkerke R^2	0.63		0.64	
	AKI and/or death H72			
Variable	H72 (OR (95% CI))	p value	H72 (OR (95% CI))	p value
Baseline mean arterial pressure	0.97 (0.94-1.01)	0.18	—	—
Baseline posology of norepinephrine	1.93 (0.70-5.32)	0.2	—	—
Baseline fluid administered	1.03 (0.99-1.07)	0.12	—	—
Baseline lactate	1.22 (0.98-1.52)	0.08	—	—
Baseline creatinine	1.01 (1.00-1.02)	0.04	1.01 (0.99-1.01)	0.06
Baseline urine output	0.65 (0.30-1.39)	0.27	—	—
Baseline TIMP2∗IGFBP7	1.21 (1.00-1.45)	0.04	1.31 (1.12-1.53)	0.001
Nagelkerke R^2	0.45		0.29	

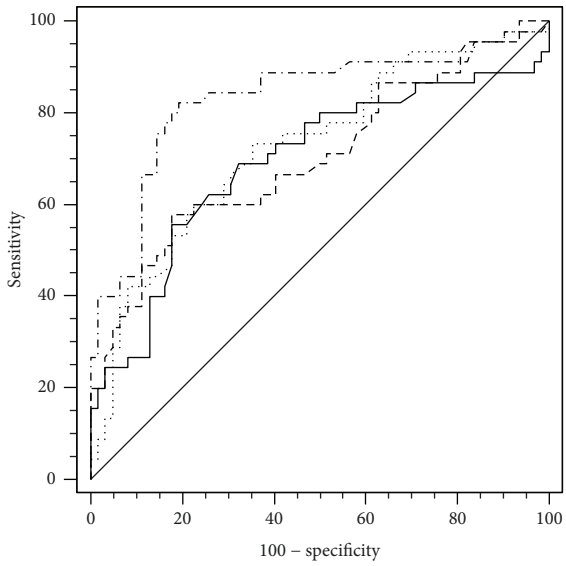
KDIGO 3 at H72 (Table 2). Those two variables were then introduced alone in the regression model, and only TIMP2∗IGFBP7 at baseline remained significantly associated with KDIGO 3 at H72 (Table 2).

The diagnostic performance of baseline creatinine and baseline TIMP2∗IGFBP7 was poor (AUC: 0.64 (0.55-0.73) and 0.69 (0.59-0.77), respectively). A baseline creatinine > 156 μmol/l predicted the KDIGO 3 level 72 hours after inclusion with a sensitivity of 47% (32-62), a specificity of 77% (65-86), a positive predictive value of 59% (42-75), and a negative predictive value of 67% (55-77). A TIMP2∗IGFBP7 > 2.08 predicted the KDIGO 3 level H72 with a sensitivity of 64% (48-77), a specificity of 75% (63-85), a positive predictive value of 65% (54-75), and a negative predictive value of 74% (66-81).

4. Discussion

In the early phase of septic shock, the urinary concentration of TIMP2∗IGFBP7 identifies the patients at high risk of progression from mild or moderate to severe AKI during the following 24 hours but not 72 hours. A test score > 2.0 $(\text{ng/ml})^2/1{,}000$ quadrupled the risk of progressing to KDIGO 3.

TIMP2 and IGFBP7 are two proteins released in the urine by renal tubular cells in response to injury. These



Variable		AUC (95% CI)	Cut off	Sensitivity	Specificity
Baseline posology of norepinephrine	——	0.69 (0.60-0.78)*	>0.51	56%	82%
Baseline creatinine	– – –	0.70 (0.61-0.79)*	>148	58%	81%
Baseline urine output	·····	0.73 (0.63-0.81)	<0.55	73%	63%
Baseline TIMP2*IGFBP7	–·–·	0.83 (0.75-0.90)	>1.92	78%	81%

Figure 3: Receiver operating curve for the diagnostic of severe AKI (KDIGO 3) within 24 hours following inclusion. $^*p < 0.05$ vs. baseline TIMP2∗IGFBP7.

molecules are released in the case of inflammation and ischaemia of tubular cells in order to block the cell cycle of adjacent tubular cells [13, 14]. Many studies have already demonstrated the relationship between high urinary TIMP2∗IGFBP7 concentrations and the risk of developing AKI [6–9, 11]. In the Topaz study conducted in 420 critically ill adults admitted to 23 participating ICUs, Bihorac et al. showed that urinary TIMP2∗IGFBP7 was an independent factor associated with AKI (KDIGO 2 or 3) within 12 hours [9]. Two different cutoff values were tested: TIMP2∗IGFBP7 > 0.3 (sensitivity of 92% and specificity of 46%) and >2.0 (sensitivity of 37% and specificity of 95%). The median TIMP2∗IGFBP7 in the AKI group was 1.6 (0.2-2.8) versus 0.3 (0.2-0.8) $(ng/ml)^2/1{,}000$ in the non-AKI group. In 50 patients undergoing cardiac surgery, Meersch et al. found that TIMP2∗IGFBP7 > 0.3 $(ng/ml)^2/1{,}000$, which was associated with the diagnosis of AKI (KDIGO≥1) (sensitivity of 80% and specificity of 83%) 4 hours after cardiac surgery [11]. The median TIMP2∗IGFBP7 4 hours after surgery in the AKI group was 1.5 versus 0.2 $(ng/ml)^2/1{,}000$ in the non-AKI group. The median urinary TIMP2∗IGFBP7 concentrations observed in our study were particularly high compared to the values reported in these previous two studies (Table 1). However, these previous studies included unselected ICU patients (only 20 to 30% of whom presented sepsis) and excluded patients with KDIGO 2 or 3 AKI. The present study only included patients with septic shock who had already developed AKI (KDIGO 1 or 2), accounting for the more severe kidney injury and the relatively high urinary TIMP2∗IGFBP7 concentrations observed. Our study is the first to specifically look at the progression from mild and moderate to severe AKI whereas others looked at none or mild AKI progression to moderate or severe AKI [9–12]. Only one study focused on the specific condition of septic shock [12]. Because inflammation is associated with the cellular expression of TIMP2 and IGFBP7 [15], it was also important to confirm the interest of TIMP2∗IGFBP7 in this setting of septic patients (one of the leading causes of AKI in ICU).

Although urinary TIMP2∗IGFBP7 concentrations appear to be an independent factor associated with the onset of severe AKI, our results show that this diagnostic value is clinically relevant only at H24 and not at H72. We hypothesized that the risk of developing KDIGO 3 AKI was related to the early urinary TIMP2∗IGFBP7 concentration reflecting the severity of the initial kidney aggression. The relationship between the initial injury and subsequent diagnostics of severe AKI probably changes with time due to additional kidney injuries (for example, the prescription of nephrotoxic drugs) or tubular cell repair. Urinary TIMP2∗IGFBP7 concentrations are early markers of tubular injury and reflect the short-term risk of severe AKI diagnostics. In contrast, serum creatinine and urine output are known to be late markers of kidney function [16]. The early tubular injury detected by the release of TIMP2∗IGFBP7 would be associated with the worsening of the kidney function. As we show here, TIMP2∗IGFBP7 seems to be poorly associated with kidney function later than 24 hours. TIMP2∗IGFBP7 may be considered only as a short-term reflection of the kidney function.

The results of this study can help to rapidly stratify the risk of progression to KDIGO 3 AKI over the next 24 hours, which could have a number of applications, particularly in clinical research. Several ongoing studies are trying to limit the development of AKI in populations at high risk of AKI. For example, nicotinamide and cellular immunotherapy are currently under study with regard to decreasing the development of AKI following cardiac surgery or in the presence of septic shock. Future clinical studies may also focus on progression to severe AKI, and our results may help investigators to select this population at highest risk of severe AKI. The treatments currently under investigation to avoid the development of AKI could then be tested to avoid further deterioration of kidney function and to promote recovery of kidney function in septic patients.

Our results should not be interpreted as a potential indication for the early initiation of RRT in this population of patients at high risk of progression to KDIGO 3, as two multicenter randomized controlled trials have shown that KDIGO 3 per se does not constitute an indication for the initiation of RRT, which depends on either an urgent indication (hyperkalemia, pulmonary edema, or severe acidosis) or prolonged anuria (>72 h) (AKIKI and IDEAL ICU). Therefore, only some of the patients identified to be at high risk of progression to KDIGO 3 finally required RRT, as in our population, TIMP2∗IGFBP7 was not associated with the need for RRT and only a small

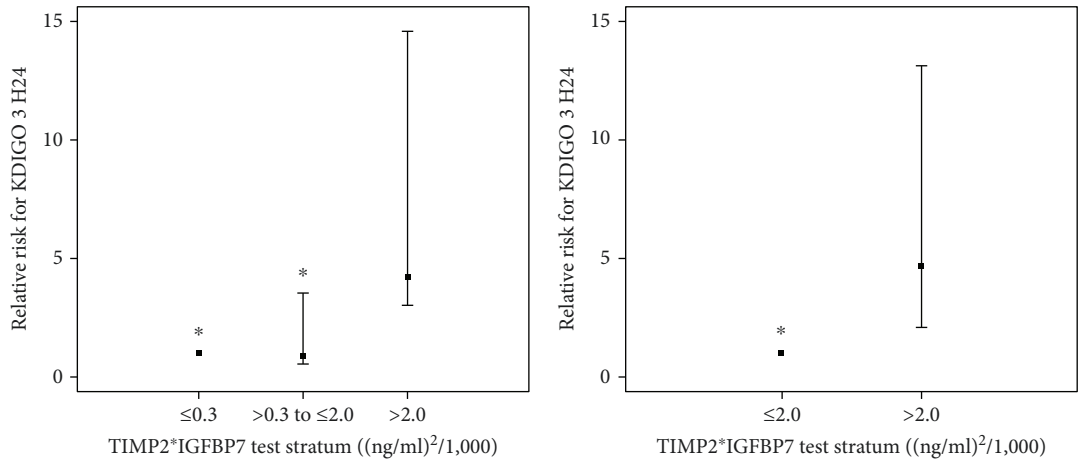


Figure 4: Relative risk of acute kidney injury (AKI) KDIGO 3 level within 24 hours (KDIGO 3 H24) in TIMP2∗IGFBP7 strata. (a) KDIGO 3 H24 risk in the stratum with TIMP2∗IGFBP7 values that are between 0.3 and 2.0 and greater than 2.0 relative to the values less than or equal to 0.3, and (b) KDIGO 3 H24 risk in the stratum with TIMP2∗IGFBP7 values that are greater than 2.0 relative to the values less than or equal to 2.0. Error bars are 95% confidence interval. $^{*}p < 0.05$ versus >2.0.

proportion of patients who progressed to KDIGO 3 finally required RRT (16/45 KDIGO 3 at H24).

Our study presents several limits. We did not record the evolution of the urine output, creatinine, or TIMP2∗IGFBP7 12 hours after inclusion which could be more sensitive to identifying high-risk patients of KDIGO 3. Only 45 patients were KDIGO 3 at H24 and 47 patients at H72 while we included, respectively, 6 and 7 variables in the logistic regression models. The individual values of TIMP2 and IGFBP7 could not be analyzed separately because the Astute 140™ Meter only provided the product of both biomarkers. Thus, the exact contribution of each biomarker could not be determined. The high number of variables included in the logistic regression models may have resulted in an overfitted effect. This emphasizes again the importance to confirm the results in a bigger cohort of patients. We used the bone criteria to define the septic shock instead of the new SESPIS 3 definition because the inclusions started before the publication of the SEPSIS3 definition. In 20 patients (10 patients KDIGO<3 H24 and 10 patients KDIGO 3 H24), no history of basal creatinine was available and we used back calculation as recommended by the KDIGO. However, a recent study has shown that back calculation of basal creatinine has moderate agreement with the AKI severity based on the measured basal creatinine, and we may have overestimated the prevalence of AKI [17]. Because it is almost impossible to determine the exact onset of sepsis and AKI in our population, our results cannot be analyzed according to the delay between the onset of AKI and the urinary TIMP2∗IGFBP7 concentrations.

5. Conclusion

In conclusion, the urinary TIMP2∗IGFBP7 concentration at the early phase of septic shock is an independent factor to identify the population at high risk of progression from mild and moderate to severe AKI over the next 24 hours but not 72 hours. A TIMP2∗IGFBP7 concentration > 2.0 $(ng/ml)^2/1{,}000$ quadruples the risk of the KDIGO 3 level within 24 hours.

Abbreviations

AKI: Acute kidney injury
AUC: Area under the curve
ICU: Intensive care unit
IGFBP7: Insulin-like growth factor-binding protein 7
KDIGO: Kidney Disease: Improving Global Outcomes
ROC: Receiver operating characteristic
RRT: Renal replacement therapy
SAPS: Simplified acute physiology score
TIMP2: Tissue inhibitor of metalloproteinase type 2.

Data Availability

The data used to support the findings of this study are included within the article.

Ethical Approval

Ethical approval was granted by the French comité de protection des personnes Nord Ouest 2 (A01392-47).

Disclosure

The abstract of this study has been presented at the 2018 European Society of Intensive Care Medicine (ESICM) Annual Congress in Paris, France.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

JM, KK, CV, and MS designed the study. JM, KK, CV, DD, LVV, DTB, MW, and LK were responsible for the patient screening and enrollment. JM, DTB, MS, and CV analyzed the data and wrote the manuscript. All authors contributed to the interpretation of the data and provided comments on the report at various stages of development. All authors approved this manuscript in its final form.

Acknowledgments

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References

[1] J. L. Vincent, J. Rello, J. Marshall et al., "International study of the prevalence and outcomes of infection in intensive care units," *JAMA*, vol. 302, no. 21, pp. 2323–2329, 2009.

[2] S. M. Bagshaw, S. Lapinsky, S. Dial et al., "Acute kidney injury in septic shock: clinical outcomes and impact of duration of hypotension prior to initiation of antimicrobial therapy," *Intensive Care Medicine*, vol. 35, no. 5, pp. 871–881, 2009.

[3] P. M. Palevsky, K. D. Liu, P. D. Brophy et al., "KDOQI US commentary on the 2012 KDIGO clinical practice guideline for acute kidney injury," *American Journal of Kidney Diseases*, vol. 61, no. 5, pp. 649–672, 2013.

[4] E. A. J. Hoste, G. Clermont, A. Kersten et al., "RIFLE criteria for acute kidney injury are associated with hospital mortality in critically ill patients: a cohort analysis," *Critical Care*, vol. 10, no. 3, article R73, 2006.

[5] M. Legrand, A. Jacquemod, E. Gayat et al., "Failure of renal biomarkers to predict worsening renal function in high-risk patients presenting with oliguria," *Intensive Care Medicine*, vol. 41, no. 1, pp. 68–76, 2015.

[6] K. Kashani, A. al-Khafaji, T. Ardiles et al., "Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury," *Critical Care*, vol. 17, no. 1, article R25, 2013.

[7] Y. Su, Z. Gong, Y. Wu, Y. Tian, and X. Liao, "Diagnostic value of urine tissue inhibitor of metalloproteinase-2 and insulin-like growth factor-binding protein 7 for acute kidney injury: a meta-analysis," *PLoS One*, vol. 12, no. 1, article e0170214, 2017.

[8] C. Liu, X. Lu, Z. Mao et al., "The diagnostic accuracy of urinary [TIMP-2]·[IGFBP7] for acute kidney injury in adults: a PRISMA-compliant meta-analysis," *Medicine*, vol. 96, no. 27, article e7484, 2017.

[9] A. Bihorac, L. S. Chawla, A. D. Shaw et al., "Validation of cell-cycle arrest biomarkers for acute kidney injury using clinical adjudication," *American Journal of Respiratory and Critical Care Medicine*, vol. 189, no. 8, pp. 932–939, 2014.

[10] M. Kimmel, J. Shi, J. Latus et al., "Association of renal stress/damage and filtration biomarkers with subsequent AKI during hospitalization among patients presenting to the emergency department," *Clinical Journal of the American Society of Nephrology*, vol. 11, no. 6, pp. 938–946, 2016.

[11] M. Meersch, C. Schmidt, H. Van Aken et al., "Urinary TIMP-2 and IGFBP7 as early biomarkers of acute kidney injury and renal recovery following cardiac surgery," *PLoS One*, vol. 9, no. 3, article e93460, 2014.

[12] M. Cuartero, J. Ballús, J. Sabater et al., "Cell-cycle arrest biomarkers in urine to predict acute kidney injury in septic and non-septic critically ill patients," *Annals of Intensive Care*, vol. 7, no. 1, p. 92, 2017.

[13] Q. H. Yang, D. W. Liu, Y. Long, H. Z. Liu, W. Z. Chai, and X. T. Wang, "Acute renal failure during sepsis: potential role of cell cycle regulation," *The Journal of Infection*, vol. 58, no. 6, pp. 459–464, 2009.

[14] R. Witzgall, D. Brown, C. Schwarz, and J. V. Bonventre, "Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the postischemic kidney. Evidence for a heterogenous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells," *The Journal of Clinical Investigation*, vol. 93, no. 5, pp. 2175–2188, 1994.

[15] J. Boonstra and J. A. Post, "Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells," *Gene*, vol. 337, pp. 1–13, 2004.

[16] S. S. Waikar, R. A. Betensky, S. C. Emerson, and J. V. Bonventre, "Imperfect gold standards for kidney injury biomarker evaluation," *Journal of the American Society of Nephrology*, vol. 23, no. 1, pp. 13–21, 2012.

[17] F. Kork, F. Balzer, A. Krannich et al., "Back-calculating baseline creatinine overestimates prevalence of acute kidney injury with poor sensitivity," *Acta Physiologica*, vol. 219, no. 3, pp. 613–624, 2017.

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Research Article

Oxidative Stress in Animal Models of Acute and Chronic Renal Failure

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Introduction. Kidney disease is a worldwide health and economic burden, with rising prevalence. The search for biomarkers for earlier and more effective disease screening and monitoring is needed. Oxidative stress has been linked to both, acute kidney injury (AKI) and chronic kidney disease (CKD). The aim of our study was to investigate whether the concentrations of systemic markers of oxidative stress and antioxidant status are affected by AKI and CKD, and to identify potential biomarkers. *Methods*. In adult male Wistar rats, AKI was induced by bilateral nephrectomy, and CKD was induced by 5/6 nephrectomy. Blood was collected 48 hours after surgery in AKI and 6 months after surgery in CKD. Advanced oxidation protein products (AOPP), thiobarbituric acid reactive substances (TBARS), advanced glycation end products (AGEs), fructosamine, total antioxidant capacity (TAC), and ferric reducing antioxidant power (FRAP) were measured. *Results*. Impaired renal function was confirmed by high concentrations of plasma creatinine and urea in AKI and CKD animals. AOPP and fructosamine were higher by 100% and 54% in AKI, respectively, and by 100% and 199% in CKD, respectively, when compared to corresponding control groups. Similarly, there was approximately a twofold increase in AGEs (by 92%) and TAC (by 102%) during AKI. In CKD, concentrations of FRAP, as an antioxidative status marker, were doubled (by 107%) when compared to the control group, but concentration of TAC, another marker of antioxidative status, did not differ between the groups. *Conclusions*. AKI and CKD led to increased systemic oxidative stress. AOPP and fructosamine could be considered potential biomarkers for both, acute and chronic kidney damage. On the other hand, AGEs, TAC, and FRAP seem to be disease specific, which could help to differentiate between acute and chronic kidney injuries. However, this needs further validation in clinical studies.

1. Introduction

Kidney disease can be classified into two types: acute kidney injury (AKI) and chronic kidney disease (CKD). The global prevalence of both forms of kidney disease is rising continuously, in part due to the aging population and in part due to a global increase in the prevalence of hypertension and diabetes. AKI is characterized by a sudden loss of kidney function within 7 days. CKD develops due to a structural or functional kidney irregularity that persists for at least 3 months. Both AKI and CKD result in the accumulation of toxic end products of nitrogen metabolism and creatinine in the blood [1–3]. Research is focused on the search for more effective and less costly therapies of AKI and end-stage CKD. More importantly, the search for screening methods of earlier detection and more effective disease monitoring is ongoing [4].

Oxidative stress is a state of imbalance between the generation of prooxidants and the number of antioxidants present in favor of prooxidants. Free radicals and nonradical oxidants alter biomolecules, mainly lipids, proteins, and

nucleic acids, ultimately leading to cell death. Mitochondria provide the main energy source for cells. In the kidney, renal tubular cells are especially rich in mitochondria, because the reabsorption of solutes is highly energy demanding. Renal tubules are particularly vulnerable to oxidative stress and damage, since mitochondria are one of the main sites of intracellular free radical production via the respiratory chain and NADPH oxidases [5–7]. Increased production of prooxidants is involved in many pathological pathways of AKI and CKD [8].

In CKD, impaired mitochondrial function and enhanced mitochondrial reactive oxygen species (ROS) has been proposed as one of the causes of elevated oxidative stress [9]. Increased production of mitochondrial ROS was shown in the kidneys of diabetic mice [10–12]. In AKI, oxidative damage and decreased antioxidant status can develop in the renal tissue due to ischemia and toxic damage [13–15]. Also, oxidative stress is a key factor in the pathogenesis of rhabdomyolysis-induced myoglobinuric AKI [16]. Another source of oxidative stress in later stages of CKD may be the presence of uremic toxins and dysregulated metabolic waste disposal [17–23]. In patients with CKD, uremia-specific risk factors, including volume expansion, chronic inflammation, anemia, or a microinflammatory state, are associated with systemic oxidative stress that in turn could cause inflammation and further tissue damage [6]. Thus, oxidative stress can affect the progression of renal disease as well in a bidirectional manner. It can aggravate inflammation, contribute to the development of fibrosis, via enhanced inflammation and trigger signaling pathways leading to renal tubular cell death. Fibrosis and inflammation might increase further formation of ROS [24]. According to the above-mentioned studies, it has been shown that oxidative stress is strongly associated with AKI and CKD and their complications. However, it is still questionable whether an elevated production of prooxidants is the only reason of oxidative stress. Uremic toxins bolster inflammation along with oxidative stress by priming polymorphonuclear leukocytes and triggering an innate immune response. Uric acid synthesis can further promote oxidative stress through the activity of xanthine oxidoreductase. On the other hand, the uric acid itself can act as an antioxidant [25–27].

Thus, oxidative stress and antioxidant status markers need to be analyzed in detail. Oxidative status is commonly assessed in the plasma by measuring the concentrations of damaged biomolecules by free radicals and other oxidants. Lipid peroxidation is routinely measured by the thiobarbituric acid-reacting substance (TBARS) method [28]. For the evaluation of protein oxidation, advanced oxidation protein products (AOPP) are assessed [29]. Advanced glycation end products (AGEs) along with fructosamine are formed as late-stage products of nonenzymatic glycation of amino groups of proteins by the carbonyl compound of sugars, and they are used as markers of carbonyl stress [30]. Total antioxidant capacity (TAC) and ferric reducing antioxidant power (FRAP) are commonly used methods for the assessment of the antioxidant status [31, 32].

To our knowledge, only very few studies describing oxidative stress and antioxidant status markers in detail in both AKI and CKD have been published. The most recent one suggests that salivary AOPP could be a suitable marker for the detection of CKD in children with 92% sensitivity and specificity [33]. Since AOPP are nonspecific, a panel of oxidative stress markers would be more helpful in the detection of CKD or AKI. Therefore, the main goal of this study was to experimentally determine the effect of acute kidney injury and chronic kidney disease on the systemic oxidative status. In addition, AKI and CKD were analyzed in parallel to search for possible differences between the two conditions, which might prove useful in the differentiation between progressive CKD and AKI as a complication of CKD or alone.

2. Methods

2.1. Animals. Twelve-week-old adult male Wistar rats were used in this experiment ($n = 40$ in total, weighing 294 ± 79 grams, Anlab, Prague, Czech Republic). Rats were housed in standard cages with wood chip bedding, in a room with an ambient temperature of $22 \pm 1°C$, 40-50% humidity, and a 12/12-hour light/dark cycle. All rats had *ad libitum* access to standard rodent chow and tap water throughout the experiment. This study was approved by the Ethics Committee of the Institute of Pathophysiology, Comenius University, and was carried out according to relevant national legislation.

2.2. Modeling of Acute Kidney Injury. Bilateral nephrectomy was performed to model AKI. Rats were divided into two groups, a bilateral nephrectomy group (BNex, $n = 11$) and a sham group (BNex sham, $n = 6$). The animals were bilaterally nephrectomised in one surgical session, as described previously [34]. Animals were anesthetized by ketamine (100 mg/kg, Richter Pharma AG, Wels, Austria) and xylazine (10 mg/kg, Ecuphar N.V., Oostkamp, Belgium) administered by intraperitoneal injection. A midline incision was made in the BNex group; the kidneys were exposed and decapsulated. Renal pedicles were tied off with a suture, and the kidneys were removed. The incision was closed with an absorbable suture. Animals in the sham group underwent sham surgery. Both kidneys of the rats in this group were decapsulated. Animals were sacrificed 48 hours after surgery, under general anesthesia. Blood was collected into EDTA-coated blood collection tubes (Sarstedt, Numbrecht, Germany) from the abdominal aorta. Plasma was obtained by centrifugation (5000 g for 5 minutes) and stored at -20°C until further analysis.

2.3. Modeling of Chronic Kidney Disease. To model CKD, 5/6 nephrectomy was conducted. Rats were divided into two groups, a 5/6 nephrectomised (5/6 Nex, $n = 14$) and a sham group (5/6 Nex sham, $n = 9$). The animals in the 5/6 Nex group underwent subtotal (5/6) nephrectomy in two surgical steps, as reported previously [35]. Briefly, animals were anesthetized by ketamine (100 mg/kg, Richter Pharma AG, Wels, Austria) and xylazine (10 mg/kg, Ecuphar N.V., Oostkamp, Belgium) administered by intraperitoneal injection. A midline incision was made on the left side, and the left kidney was exposed. The kidney was decapsulated, and the upper and lower kidney poles were removed. Bleeding was stopped

by Gelaspon (Chauvin Ankerpharm GmbH, Rudolstadt, Germany), and the incision was closed with an absorbable suture. After the recovery period (14 days later), a similar incision was made on the right side, the renal vessels were ligated, and the decapsulated kidney was removed. Animals in the 5/6 Nex sham group underwent sham surgery. The kidneys of the rats in this group were decapsulated at the time of the first surgery. Animals were sacrificed 6 months after surgery under general anesthesia. Animals were placed in metabolic cages (4 hours) for urine collection. Plasma samples were obtained in the same manner as described in the AKI model.

2.4. Biochemical Analysis. Three hundred and fifty microliters of plasma were used to measure plasma creatinine, urea, and albuminuria concentrations using the Biolis 24i Premium automated clinical analyzer (Tokyo Boeki Medical System Ltd., Tokyo, Japan) [36]. The principle of urea measurement is based on the hydrolysis of urea by urease to form ammonium and carbonate. The second step is based on the reaction 2-oxoglutarate that reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH. This reaction produces L-glutamate [37]. The measurement of creatinine is based on its reaction with picric acid in alkaline conditions that results in a reddish complex [38]. The plasma concentrations of kidney injury molecule 1 (KIM-1) were measured using the commercial rat KIM-1 ELISA kit (R&D Systems Inc., Abingdon, UK) according to the manufacturer's protocol.

2.5. Oxidative Stress and Antioxidant Status Analysis. Markers of oxidative stress and antioxidant status were measured in plasma samples using a Synergy H1 multimode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

TBARS were measured by pipetting 20 μl of samples or standards (1,1,3,3-tetraethoxypropane), 30 μl of distilled water, 20 μl of 0.67% thiobarbituric acid, and 20 μl of glacial acetic acid in a microtiter plate. The plates were mixed and incubated for 45 minutes at 95°C. 100 μl of n-butanol was added into the samples, and the plates were centrifuged (2000 g/10 min/4°C). Seventy microliters of the upper phase was transferred into a new microtiter plate, and fluorescence was measured at $_{ex}$ = 515 nm and λ_{em} = 535 nm. AOPP was assessed by mixing 200 μl of samples and standards (chloramine T mixed with potassium iodide) with 20 μl of glacial acetic acid for 2 minutes. Absorbance was measured at 340 nm. AGEs were measured by pipetting 20 μl of sample or standards (AGE-BSA) and 180 μl of PBS into a dark microtiter plate, vortexing and measuring fluorescence at $_{ex}$ = 370 nm and λ_{em} = 440 nm. For fructosamine measurement, 20 μl of the samples and standards (16 mmol/l 1-deoxy-morpholino-D-fructose) was mixed with 100 μl of 0.25 mmol/l nitroblue tetrazolium containing 1 mmol/l nitroblue tetrazolium and 0.1 mol/l sodium carbonate buffer (pH = 10.35). Samples were incubated at 37°C for 15 minutes. Absorbance was measured at 530 nm.

For TAC measurement, samples were mixed with acetate buffer (pH = 5.8). Absorbance was measured at 660 nm as blank. When the ABTS solution (2.2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid with acetate buffer)) was added, the absorbance was measured again at 660 nm. The blank absorbance values were subtracted from the values obtained by the second measurement. For FRAP assessment, 200 μl of warmed (37°C) FRAP reagent (containing acetate buffer (pH = 3.6), tripyridyl-s-triazine, $FeCl_3 * 6H_2O$, and water) was pipetted into a microtiter plate, and absorbance was measured as blank. Afterwards, 20 μl of samples and standards (100 mmol/l $FeSO_4 * 7H_2O$) was added. Absorbance was measured again at 530 nm. The blank absorbance values were subtracted from the values obtained by the second measurement [39].

For the measurement of proteins, 10 μl of the samples and standards (bovine serum albumin) was mixed with 200 μl of the working solution (bicinchoninic acid and copper sulphate, 49 : 1 ratio, respectively). The plate was incubated at 37°C for 30 minutes. After cooling, absorbance was measured at 562 nm. All markers measured in plasma were normalized to plasma protein concentrations. Markers measured in urine were normalized to urinary creatinine concentrations.

2.6. Statistical Analysis. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses. After testing for normality with the D'Agostino-Pearson omnibus test, data were analyzed with the Mann–Whitney U test. The Spearman correlation test and linear regression were used to evaluate the linear associations between quantitative variables. A value of $p < 0.05$ was considered statistically significant.

3. Results

Concentrations of creatinine in the plasma of the BNex group were 20-fold higher when compared to the BNex sham group (Figure 1(a); $U = 0$, $p < 0.001$). Blood urea in the BNex group was significantly higher when compared to the BNex sham group by 484% (Figure 1(b); $U = 0$, $p < 0.001$). Plasma creatinine concentrations in the 5/6 Nex group were significantly higher than in the 5/6 Nex sham group by 48% (Figure 1(c); $U = 18$, $p < 0.01$). Blood urea was 2-fold higher in the 5/6 Nex group than in the 5/6 Nex sham group (Figure 1(d); $U = 10$, $p < 0.001$). One animal in the BNex group did not show elevated levels of plasma creatinine (37.56 μmol/l), nor urea (11.70 mmol/l), which would comply with the modeling of AKI and was thus removed from further analyses.

There were no differences between the BNex and the BNex sham groups in TBARS concentrations (Figure 2(a)). In plasma TBARS, no significant differences were found between the 5/6 Nex and the 5/6 Nex sham groups either (Figure 3(a)). Plasma AOPP concentrations were 2-fold higher in the BNex group, when compared to the BNex sham group (Figure 2(b); $U = 9$, $p < 0.05$). In plasma AOPP, a significant difference was found between the 5/6 Nex and the 5/6 Nex sham groups, the first being higher by 102% (Figure 3(b); $U = 28$, $p < 0.05$).

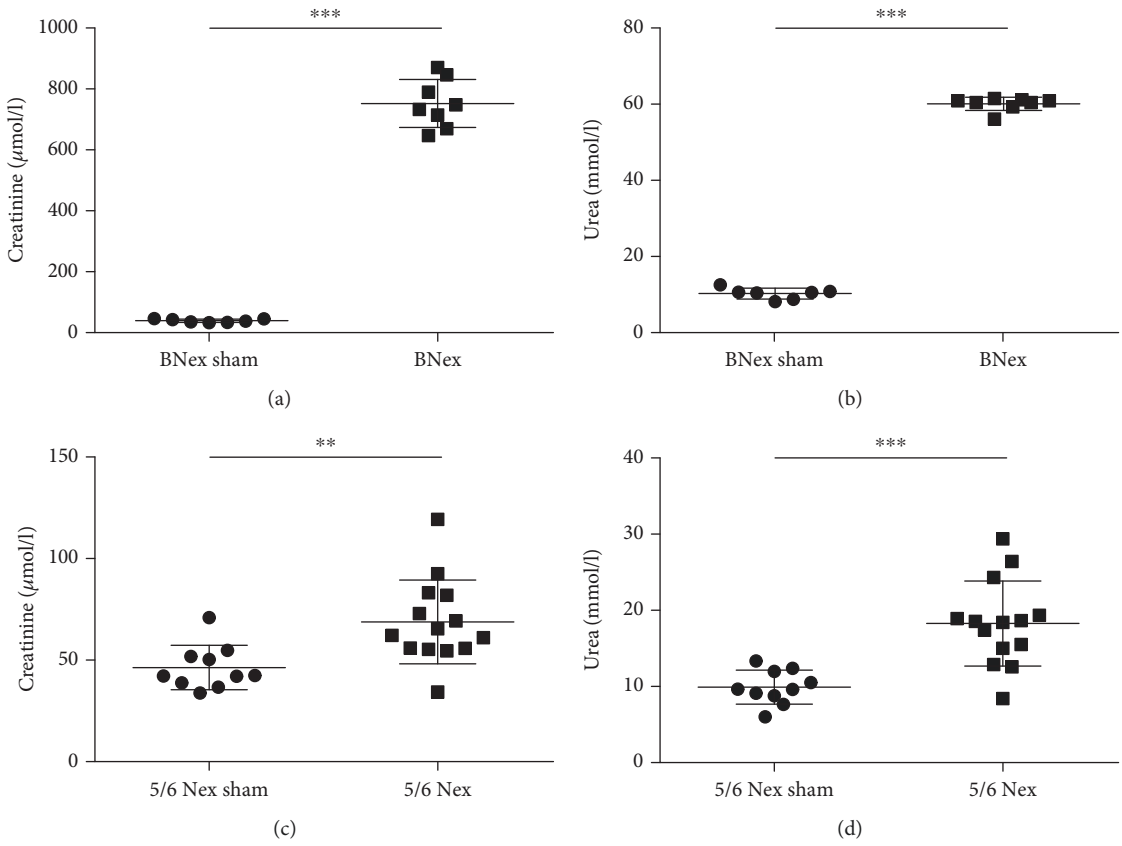

Figure 1: Renal function parameters in rats with acute and chronic kidney injury and sham-operated rats. (a) Plasma creatinine in bilaterally nephrectomised (BNex) and sham (BNex sham) rats, (b) plasma urea in BNex and BNex sham rats, (c) plasma creatinine in 5/6 nephrectomised (5/6 Nex) and sham (5/6 Nex sham) rats, and (d) plasma urea in 5/6 Nex and 5/6 Nex sham rats. $^{**}p < 0.01$, $^{***}p < 0.001$.

AGE concentrations were 2-fold higher in the plasma of the BNex group, when compared to the BNex sham group (Figure 2(c); $U = 0$, $p < 0.01$). No significant differences were found in plasma AGE concentration between the 5/6 Nex and the 5/6 Nex sham groups (Figure 3(c)). Fructosamine concentrations differed significantly between the BNex and the BNex sham group as well, the BNex group being higher by 54% (Figure 2(d); $U = 4$, $p < 0.01$). In plasma fructosamine, a significant difference was found between the 5/6 Nex and the 5/6 Nex sham groups. The 5/6 Nex group had 3-fold higher fructosamine concentrations (Figure 3(d); $U = 10$, $p < 0.01$). Between the BNex and the BNex sham groups, there was a significant difference in TAC, the BNex being 2-fold higher (Figure 2(e); $U = 5$, $p < 0.05$). TAC concentrations in plasma did not differ between the 5/6 Nex and the 5/6 Nex sham groups (Figure 3(e)). There were no differences between the BNex and the BNex sham groups in FRAP concentrations (Figure 2(f)). In plasma FRAP, a significant difference was found between the 5/6 Nex and the 5/6 Nex sham groups, the first being 2-fold higher (Figure 3(f); $U = 22$, $p < 0.05$).

Additionally, to the plasma concentrations, TBARS, AOPP, fructosamine, and FRAP were all measurable in the urine of 5/6 Nex rats. Of these, AOPP differed most significantly from the sham group. AOPP in 5/6 Nex were 2-fold higher in urine when compared to 5/6 Nex sham group (Figure 4(b); $U = 7$, $p < 0.001$). TBARS, fructosamine, and FRAP in urine were increased in the 5/6 Nex group by approximately 50% (Figures 4(a), 4(c), and 4(d); $U = 40$, 34, and 32, respectively, $p < 0.05$ for all three markers) when compared to the control group.

In 5/6 Nex as a model of CKD, urinary AOPP significantly and positively correlated with urinary KIM-1 ($r = 0.70$; $p < 0.05$). Also, urinary fructosamine and FRAP significantly correlated with ACR ($r = 0.72$ and $r = 0.82$; $p < 0.05$, respectively) and albuminuria ($r = 0.62$ and $r = 0.82$; $p < 0.05$, respectively), but neither with plasma nor with urinary KIM-1 (Table 1).

4. Discussion

The results of this experiment confirmed the association between elevated systemic oxidative stress and acute and chronic renal failure. Although proteins are relatively resistant to damage by prooxidants, AOPP concentrations were 2-fold higher in rats with both acute and chronic renal failure compared to their sham-operated counterparts. A 2-fold increase in AOPP was present not only in plasma but also in the urine of CKD rats. These findings are in line with previous studies in AKI and CKD patients [40, 41]. It has been proposed that AOPP not only are associated with CKD but also have a pathogenic role in CKD progression

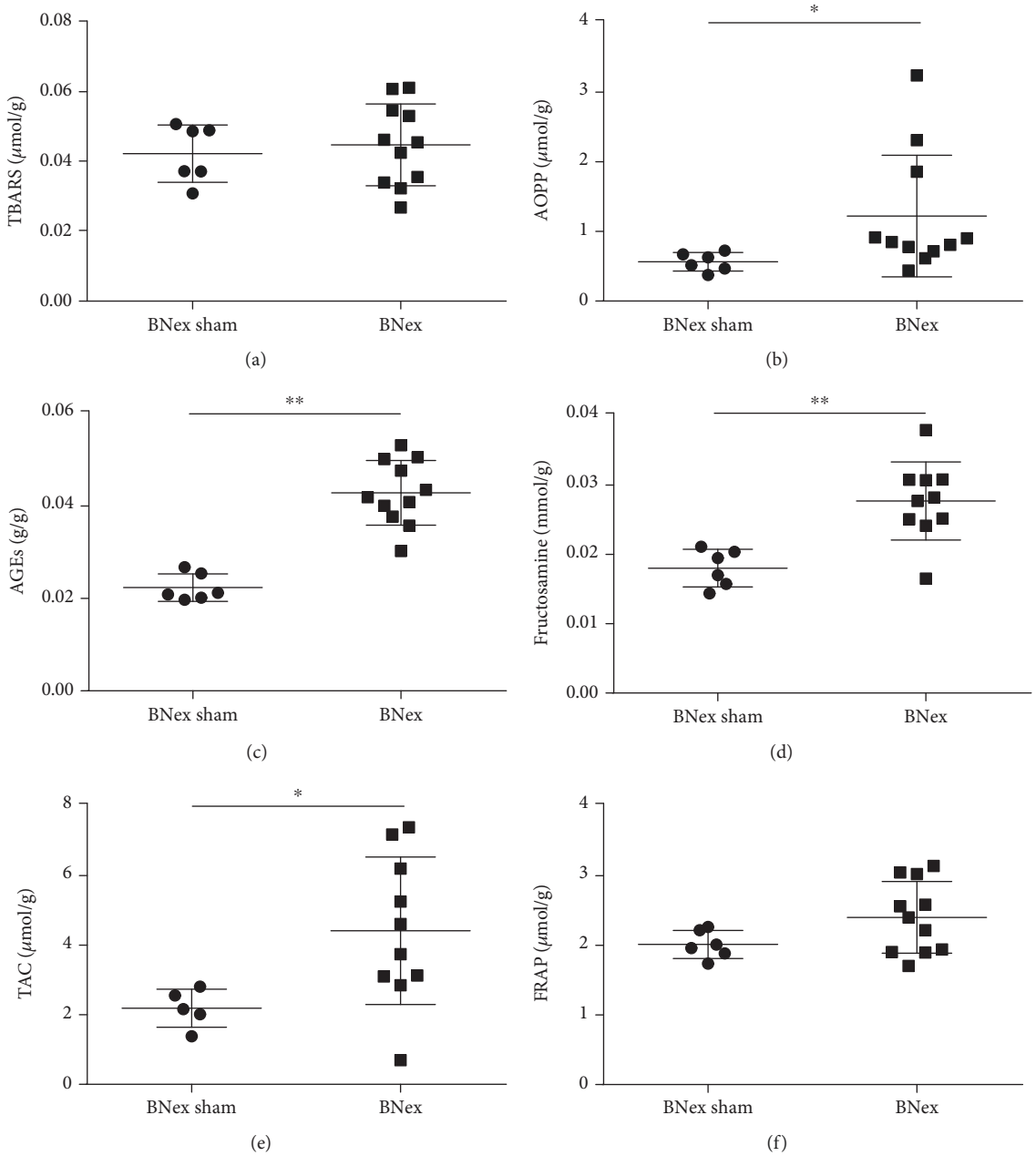

Figure 2: Oxidative stress and antioxidant status markers in the plasma of bilaterally nephrectomised (BNex) and sham (BNex sham) rats. (a) TBARS: thiobarbituric acid reactive substances, (b) AOPP: advanced oxidation protein products, (c) AGEs: advanced glycation end products, (d) fructosamine, (e) TAC: total antioxidant capacity, and (f) FRAP: ferric reducing antioxidant power. $^{*}p < 0.05$, $^{**}p < 0.01$.

through cellular and molecular mechanisms. These mechanisms include triggering a cascade of signaling events that lead to superoxide generation, NF-κB activation, the overproduction of extracellular matrix, apoptosis of podocytes, endothelial inflammation, and monocyte activation [42, 43]. Moreover, elevated AOPP were found to be associated with poor prognosis of patients with IgA nephropathy [44]. On the other hand, during CKD, the excessive glomerular protein leakage might contribute to the absolute numbers of AOPP.

This study found no differences between 5/6 Nex or BNex animals and the sham groups in oxidized lipid concentrations measured by TBARS in plasma, contrary to other studies. A previous study showed that products of lipid peroxidation could be increasing in AKI in a time-dependent manner. It could be argued that these damaged lipids would have increased should the experiment lasted longer, although, in the mentioned studies, lipid peroxidation products were already significantly higher after 48 hours in rats with AKI [45, 46]. On the other hand, urinary TBARS were higher in CKD rats, suggesting their clearance from circulation despite reduced renal function. Unfortunately, due to the anuria of the BNex rats, we were unable to determine the urinary levels of TBARS in the acute setting.

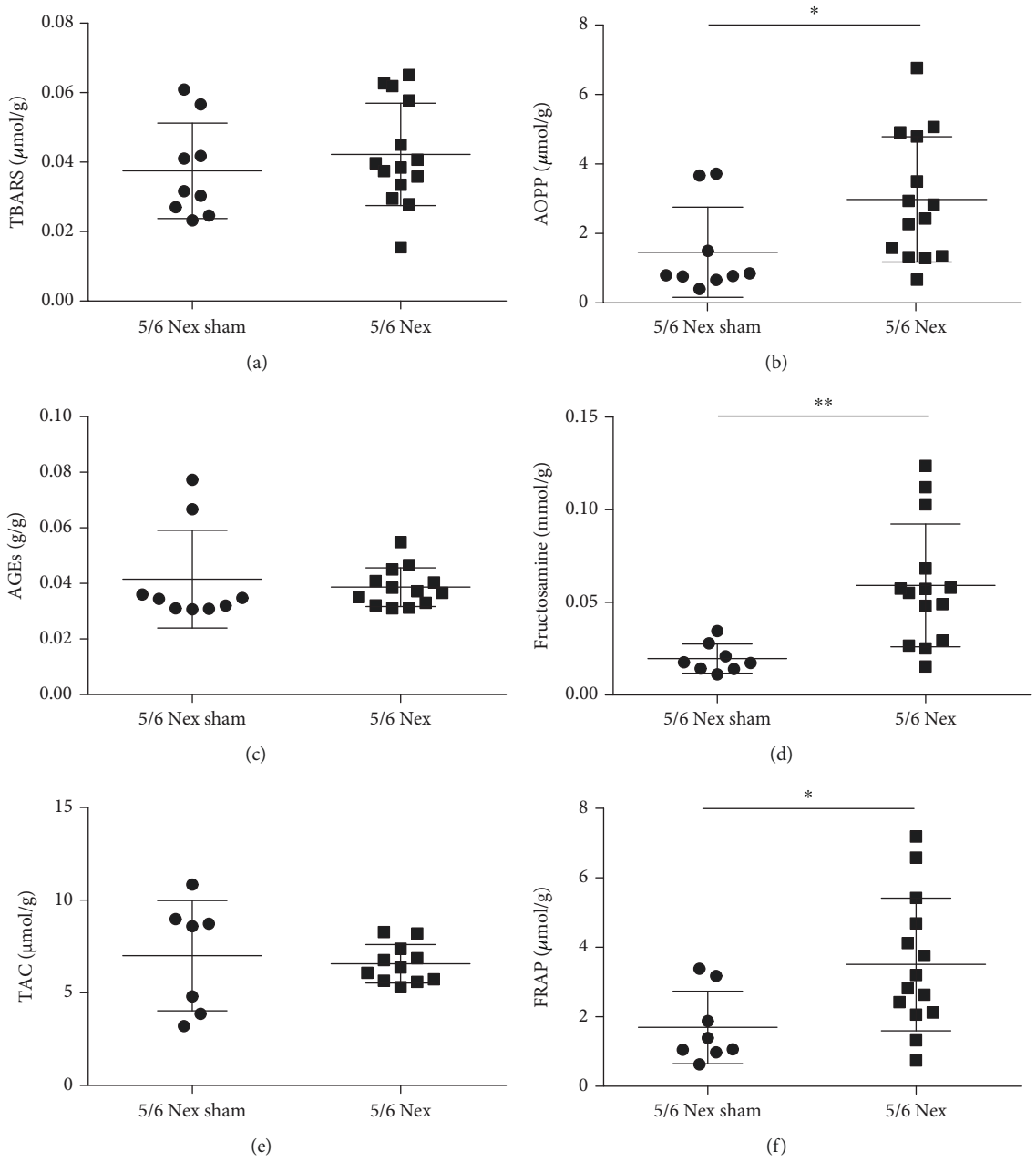

Figure 3: Oxidative stress and antioxidant status markers in the plasma of 5/6 nephrectomised (5/6 Nex) and sham (5/6 Nex sham) rats. (a) TBARS: thiobarbituric acid reactive substances, (b) AOPP: advanced oxidation protein products, (c) AGEs: advanced glycation end products, (d) fructosamine, (e) TAC: total antioxidant capacity, and (f) FRAP: ferric reducing antioxidant power. $^{*}p < 0.05$, $^{**}p < 0.01$.

Products of carbonyl stress, such as fructosamine and AGEs, are produced during aging and accumulate in circulation and tissues. Kidneys play an important part in their disposal, and fructosamine and AGEs have been shown to be elevated when kidney function is compromised [47, 48]. The results of this experiment showed that the concentration of fructosamine was higher in AKI and CKD, but AGEs were higher only in the AKI model. This could indicate that the remaining kidney function in the mild 5/6 Nex model of CKD was sufficient to filter AGEs from circulation. The reasons of the accumulation of AGEs during uremia are not fully understood. Increased concentration of circulating AGEs in patients with kidney diseases may result from an increased production due to uremia-related consequences or decreased renal disposal [49]. According to previous results, low molecular weight AGEs are easily removed from the body by renal clearance [50]. Thus, it seems that a partial maintenance of low molecular weight AGE disposal by the damaged kidneys prevented the elevation of plasma AGE concentrations in 5/6 Nex. However, it has also been shown that one fraction of plasma AGEs is linked to binding proteins, mainly albumin in uremic patients. Glomerular filtration or dialysis does not remove these proteins. Thus, accumulation of higher molecular weight AGEs could not be explained by decreased

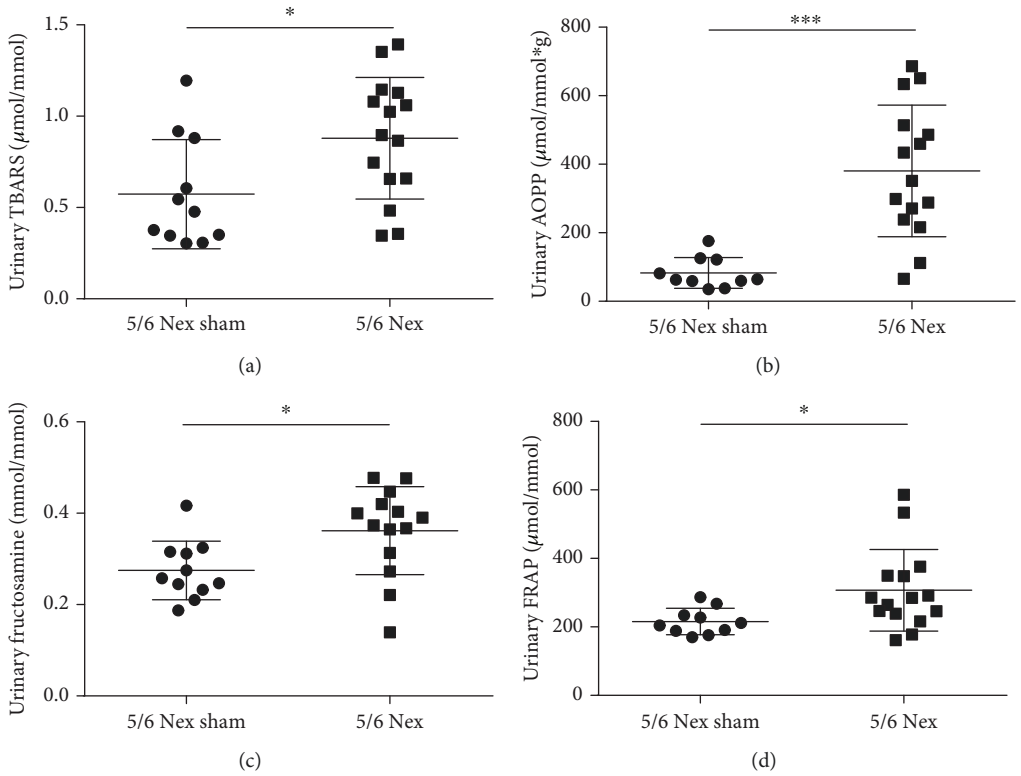

Figure 4: Oxidative stress and antioxidant status markers in the urine of 5/6 nephrectomised (5/6 Nex) and sham (5/6 Nex sham) rats. (a) TBARS: thiobarbituric acid reactive substances, (b) AOPP: advanced oxidation protein products, (c) fructosamine, and (d) FRAP: ferric reducing antioxidant power. $^{*}p < 0.05$, $^{***}p < 0.001$.

Table 1: Correlation coefficients (Spearman) between plasma markers, thiobarbituric acid reactive substances (TBARS), advanced oxidation protein products (AOPP), advanced glycation end products (AGEs), fructosamine, total antioxidant capacity (TAC), and ferric reducing antioxidant power (FRAP), and urinary markers, TBARS, AOPP, fructosamine, FRAP, plasma creatinine, blood urea nitrogen (BUN), urinary kidney injury molecule-1 (uKIM-1), plasma kidney injury molecule-1 (pKIM-1), albumin/creatinine ratio of urine (ACR), and albuminuria in 5/6 nephrectomised (CKD model) and sham rats.

	Plasma creatinine (μmol/l)	BUN (mmol/l)	pKIM-1 (pg/ml)	uKIM-1 (pg/ml)	ACR (g/mol)	Albuminuria (mg/day)
Plasma markers of OS						
TBARS (μmol/g)	-0.42 n.s.	-0.38 n.s.	0.06 n.s.	-0.30 n.s.	0.33 n.s.	0.39 n.s.
AOPP (μmol/g)	-0.12 n.s.	0.05 n.s	-0.15 n.s.	-0.05 n.s.	-0.07 n.s.	-0.08 n.s.
AGEs (g/g)	-0.60*	-0.38 n.s.	-0.38 n.s.	-0.62*	-0.32 n.s	-0.11 n.s.
Fructosamine (mmol/g)	0.10 n.s.	0.17 n.s.	-0.43 n.s.	-0.05 n.s.	-0.06 n.s.	0.07 n.s.
TAC (μmol/g)	0.01 n.s.	0.10 n.s.	0.28 n.s.	0.17 n.s.	0.0 n.s.	-0.08 n.s.
FRAP (μmol/g)	0.07 n.s.	0.10 n.s.	0.06 n.s.	0.12 n.s.	0.14 n.s.	0.12 n.s.
Urinary markers of OS						
TBARS (μmol/mmol)	0.23 n.s.	0.44 n.s.	-0.06 n.s.	0.26 n.s.	0.30 n.s.	0.01 n.s.
AOPP (μmol/mmol∗g)	0.23 n.s.	0.06 n.s.	0.38 n.s.	0.70*	0.51 n.s.	-0.10 n.s.
Fructosamine (mmol/mmol)	-0.31 n.s.	-0.32 n.s.	0.13 n.s.	0.17 n.s.	0.72*	0.62*
FRAP (μmol/mmol)	-0.33 n.s.	-0.28 n.s.	0.49 n.s.	0.32 n.s.	0.82**	0.69*

$^{*}p < 0.05$, $^{**}p < 0.01$.

renal clearance [51]. In addition, the elevated urinary levels of fructosamine in 5/6 Nex rats confirm their clearance via the kidneys.

The results also showed that the concentrations of antioxidant status markers were higher in the 5/6 Nex and BNex groups than in controls—more precisely, FRAP was higher in

the 5/6 Nex group, and TAC was found to be elevated in the BNex group when compared to sham animals. These results are in line with a different study showing higher TAC in a rat model of AKI than in sham-operated animals [52]. Another study involving CKD patients has shown higher FRAP concentrations in patients than in controls [53]. In addition, urinary FRAP levels were also elevated in 5/6 Nex, confirming the situation observed in plasma. Although we cannot fully explain the difference between AKI and CKD, this could be potentially used as a differentiating marker between AKI and CKD. It is assumed that the antioxidant activity rose as an answer to present systemic oxidative stress in both disease states. Paradoxically, the antioxidant status may increase in renal disease because of the accumulation of uremic toxins with scavenging capacity, such as indole derivatives and hippurate [48, 52]. In addition, the difference might be the result of the differences of the measured substances between the two methods. While TAC includes the SH/thiol groups present in proteins and reduced glutathione, these are not detected by the FRAP method. Due to the complete anuria in the BNex rats compared to the known proteinuria in the 5/6 Nex model, the higher TAC in BNex might be attributed to a higher amount of small molecular weight proteins in the plasma of BNex rats [54].

To further address the applicability of the oxidative stress markers in renal disease, we correlated them with the standard plasmatic and urinary markers of renal function and injury.

In CKD, we found a negative correlation between plasma AGEs vs. plasma creatinine and uKIM-1. Whether this observation is of any prognostic or diagnostic value is currently unknown. In the urine, there was a positive correlation between AOPP and uKIM-1, suggesting that urinary AOPP might closely reflect proximal tubular injury. On the other hand, urinary fructosamine and FRAP correlated with ACR and albuminuria, but not with uKIM-1, suggesting that they might more closely reflect glomerular injury. However, further studies are needed to prove these assumptions.

The limitation of this study is that the urinary concentration of oxidative stress and antioxidant status markers could not be assessed in BNex rats, due to anuria which is typical for this model [55]. Further studies will be aimed at the models with residual kidney function, which are not completely anuric.

In conclusion, this study confirms the association of AKI and CKD with elevated systemic oxidative stress markers. Furthermore, to our knowledge, this study is the first to describe this particular set of oxidative stress and antioxidant status markers measured parallel in AKI and CKD. It shows that oxidative damage to proteins is apparent in both disease states in plasma and in urine. Nevertheless, we do not report elevated lipid peroxidation in kidney disease in plasma, neither in AKI nor in CKD. Interestingly, in the urine, we found elevated markers of lipid peroxidation in CKD, which might suggest urine as a superior fluid for the determination of lipid peroxidation in CKD. Carbonyl stress is apparent by an increase in fructosamine in both disease states, but the remnant kidney function in CKD appears to be sufficient to excrete AGEs of lower molecular weight. Antioxidant status can also increase in AKI and CKD, likely due to an increase in uremic toxins with antioxidant capacity. However different antioxidant systems probably take part in increased oxidative stress during CKD and AKI. Future studies should be focused on AOPP and fructosamine as potential biomarkers, and data on their dynamics and urinary concentrations in AKI should be gathered. Further clarification of different antioxidant mechanisms during AKI and CKD is needed.

Data Availability

The Excel or GraphPad data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] J. Costa, R. S. Crausman, and M. S. Weinberg, "Acute and chronic renal failure," *Journal of the American Podiatric Medical Association*, vol. 94, no. 2, pp. 168–176, 2004.

[2] W. G. Couser, G. Remuzzi, S. Mendis, and M. Tonelli, "The contribution of chronic kidney disease to the global burden of major noncommunicable diseases," *Kidney International*, vol. 80, no. 12, pp. 1258–1270, 2011.

[3] R. Bellomo, J. A. Kellum, and C. Ronco, "Acute kidney injury," *The Lancet*, vol. 380, no. 9843, pp. 756–766, 2012.

[4] O. J. Wouters, D. J. O'Donoghue, J. Ritchie, P. G. Kanavos, and A. S. Narva, "Early chronic kidney disease: diagnosis, management and models of care," *Nature Reviews Nephrology*, vol. 11, no. 8, pp. 491–502, 2015.

[5] A. Eirin, A. Lerman, and L. O. Lerman, "The emerging role of mitochondrial targeting in kidney disease," in *Pharmacology of Mitochondria. Handbook of Experimental Pharmacology, vol 240*, H. Singh and S. S. Sheu, Eds., pp. 229–250, Springer, Cham, 2016.

[6] F. Locatelli, B. Canaud, K.-U. Eckardt, P. Stenvinkel, C. Wanner, and C. Zoccali, "Oxidative stress in end-stage renal disease: an emerging threat to patient outcome," *Nephrology Dialysis Transplantation*, vol. 18, no. 7, pp. 1272–1280, 2003.

[7] H. Sies, "Oxidative stress: oxidants and antioxidants," *Experimental Physiology*, vol. 82, no. 2, pp. 291–295, 1997.

[8] H. Yaribeygi, F. R. Farrokhi, R. Rezaee, and A. Sahebkar, "Oxidative stress induces renal failure: a review of possible molecular pathways," *Journal of Cellular Biochemistry*, vol. 119, no. 4, pp. 2990–2998, 2018.

[9] D. L. Galvan, N. H. Green, and F. R. Danesh, "The hallmarks of mitochondrial dysfunction in chronic kidney disease," *Kidney International*, vol. 92, no. 5, pp. 1051–1057, 2017.

[10] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.

[11] W. Wang, Y. Wang, J. Long et al., "Mitochondrial fission triggered by hyperglycemia is mediated by ROCK1 activation in podocytes and endothelial cells," *Cell Metabolism*, vol. 15, no. 2, pp. 186–200, 2012.

[12] B. P. Dieter, R. Z. Alicic, R. L. Meek, R. J. Anderberg, S. K. Cooney, and K. R. Tuttle, "Novel therapies for diabetic kidney disease: storied past and forward paths," *Diabetes Spectrum*, vol. 28, no. 3, pp. 167–174, 2015.

[13] M. S. Paller, J. R. Hoidal, and T. F. Ferris, "Oxygen free radicals in ischemic acute renal failure in the rat," *Journal of Clinical Investigation*, vol. 74, no. 4, pp. 1156–1164, 1984.

[14] R. Baliga, N. Ueda, P. D. Walker, and S. V. Shah, "Oxidant mechanisms in toxic acute renal failure," *Drug Metabolism Reviews*, vol. 31, no. 4, pp. 971–997, 1999.

[15] J. M. Dennis and P. K. Witting, "Protective role for antioxidants in acute kidney disease," *Nutrients*, vol. 9, no. 7, 2017.

[16] S. Ustundag, S. Sen, O. Yalcin, S. Ciftci, B. Demirkan, and M. Ture, "L-Carnitine ameliorates glycerol-induced myoglobinuric acute renal failure in rats," *Renal Failure*, vol. 31, no. 2, pp. 124–133, 2009.

[17] F. Martinon, V. Pétrilli, A. Mayor, A. Tardivel, and J. Tschopp, "Gout-associated uric acid crystals activate the NALP3 inflammasome," *Nature*, vol. 440, no. 7081, pp. 237–241, 2006.

[18] I. Sakamaki, K. Inai, Y. Tsutani, T. Ueda, and H. Tsutani, "Binding of monosodium urate crystals with idiotype protein efficiently promote dendritic cells to induce cytotoxic T cells," *Cancer Science*, vol. 99, no. 11, pp. 2268–2273, 2008.

[19] Y. Amaya, K. Yamazaki, M. Sato, K. Noda, T. Nishino, and T. Nishino, "Proteolytic conversion of xanthine dehydrogenase from the NAD-dependent type to the O2-dependent type. Amino acid sequence of rat liver xanthine dehydrogenase and identification of the cleavage sites of the enzyme protein during irreversible conversion by trypsin," *Journal of Biological Chemistry*, vol. 265, no. 24, pp. 14170–14175, 1990.

[20] T. Nishino, K. Okamoto, Y. Kawaguchi et al., "Mechanism of the conversion of xanthine dehydrogenase to xanthine oxidase," *Journal of Biological Chemistry*, vol. 280, no. 26, pp. 24888–24894, 2005.

[21] E. Peuchant, M. A. Carbonneau, L. Dubourg et al., "Lipoperoxidation in plasma and red blood cells of patients undergoing haemodialysis: vitamins A, E, and iron status," *Free Radical Biology & Medicine*, vol. 16, no. 3, pp. 339–346, 1994.

[22] J. Himmelfarb, M. E. McMenamin, G. Loseto, and J. W. Heinecke, "Myeloperoxidase-catalyzed 3-chlorotyrosine formation in dialysis patients," *Free Radical Biology & Medicine*, vol. 31, no. 10, pp. 1163–1169, 2001.

[23] P. Jackson, C. M. Loughrey, J. H. Lightbody, P. T. McNamee, and I. S. Young, "Effect of hemodialysis on total antioxidant capacity and serum antioxidants in patients with chronic renal failure," *Clinical Chemistry*, vol. 41, 8 Part 1, pp. 1135–1138, 1995.

[24] K. Hosohata, "Role of oxidative stress in drug-induced kidney injury," *International Journal of Molecular Sciences*, vol. 17, no. 11, 2016.

[25] R. Mohandas and R. J. Johnson, "Uric acid levels increase risk for new-onset kidney disease," *Journal of the American Society of Nephrology*, vol. 19, no. 12, pp. 2251–2253, 2008.

[26] D.-H. Kang and S.-K. Ha, "Uric acid puzzle: dual role as anti-oxidantand pro-oxidant," *Electrolytes & Blood Pressure*, vol. 12, no. 1, pp. 1–6, 2014.

[27] Y. Y. Sautin and R. J. Johnson, "Uric acid: the oxidant-antioxidant paradox," *Nucleosides, Nucleotides and Nucleic Acids*, vol. 27, no. 6-7, pp. 608–619, 2008.

[28] A. M. Jentzsch, H. Bachmann, P. Fürst, and H. K. Biesalski, "Improved analysis of malondialdehyde in human body fluids," *Free Radical Biology & Medicine*, vol. 20, no. 2, pp. 251–256, 1996.

[29] V. Witko-Sarsat, M. Friedlander, C. Capeillère-Blandin et al., "Advanced oxidation protein products as a novel marker of oxidative stress in uremia," *Kidney International*, vol. 49, no. 5, pp. 1304–1313, 1996.

[30] M. Brownlee, A. Cerami, and H. Vlassara, "Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications," *The New England Journal of Medicine*, vol. 318, no. 20, pp. 1315–1321, 1988.

[31] I. F. F. Benzie and J. J. Strain, "The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay," *Analytical Biochemistry*, vol. 239, no. 1, pp. 70–76, 1996.

[32] O. Erel, "A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation," *Clinical Biochemistry*, vol. 37, no. 4, pp. 277–285, 2004.

[33] M. Maciejczyk, J. Szulimowska, A. Skutnik et al., "Salivary biomarkers of oxidative stress in children with chronic kidney disease," *Journal of Clinical Medicine*, vol. 7, no. 8, 2018.

[34] T. S. Hoke, I. S. Douglas, C. L. Klein et al., "Acute renal failure after bilateral nephrectomy is associated with cytokine-mediated pulmonary injury," *Journal of the American Society of Nephrology*, vol. 18, no. 1, pp. 155–164, 2007.

[35] L. Tóthová, J. Bábíčková, V. Borbélyová, B. Filová, K. Šebeková, and J. Hodosy, "Chronic renal insufficiency does not induce behavioral and cognitive alteration in rats," *Physiology & Behavior*, vol. 138, pp. 133–140, 2015.

[36] M. Hashimoto, M. Katakura, T. Nabika et al., "Effects of hydrogen-rich water on abnormalities in a SHR.Cg-*Lepr*^{cp}/NDmcr rat - a metabolic syndrome rat model," *Medical Gas Research*, vol. 1, no. 1, p. 26, 2011.

[37] P. S. Francis, S. W. Lewis, and K. F. Lim, "Analytical methodology for the determination of urea: current practice and future trends," *TrAC Trends in Analytical Chemistry*, vol. 21, no. 5, pp. 389–400, 2002.

[38] C. Slot, "Plasma creatinine determination a new and specific Jaffe reaction method," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 17, no. 4, pp. 381–387, 1965.

[39] L. Tóthová, J. Hodosy, K. Mettenburg et al., "No harmful effect of different Coca-Cola beverages after 6 months of intake on rat testes," *Food and Chemical Toxicology*, vol. 62, pp. 343–348, 2013.

[40] P. S. Tucker, V. J. Dalbo, T. Han, and M. I. Kingsley, "Clinical and research markers of oxidative stress in chronic kidney disease," *Biomarkers*, vol. 18, no. 2, pp. 103–115, 2013.

[41] P. Lentini, M. de Cal, D. Cruz et al., "The role of advanced oxidation protein products in intensive care unit patients with acute kidney injury," *Journal of Critical Care*, vol. 25, no. 4, pp. 605–609, 2010.

[42] V. Witko-Sarsat, M. Friedlander, T. Nguyen Khoa et al., "Advanced oxidation protein products as novel mediators

of inflammation and monocyte activation in chronic renal failure," *The Journal of Immunology*, vol. 161, no. 5, pp. 2524–2532, 1998.

[43] W. Cao, F. F. Hou, and J. Nie, "AOPPs and the progression of kidney disease," *Kidney International Supplements*, vol. 4, no. 1, pp. 102–106, 2014.

[44] B. Descamps-Latscha, V. Witko-Sarsat, T. Nguyen-Khoa et al., "Early prediction of IgA nephropathy progression: proteinuria and AOPP are strong prognostic markers," *Kidney International*, vol. 66, no. 4, pp. 1606–1612, 2004.

[45] S. Owada, T. Maeba, Y. Sugano et al., "Spherical carbon adsorbent (AST-120) protects deterioration of renal function in chronic kidney disease rats through inhibition of reactive oxygen species production from mitochondria and reduction of serum lipid peroxidation," *Nephron Experimental Nephrology*, vol. 115, no. 4, pp. e101–e111, 2010.

[46] H. Ilhan, M. Eroglu, V. Inal et al., "Hyperbaric oxygen therapy alleviates oxidative stress and tissue injury in renal ischemia/reperfusion injury in rats," *Renal Failure*, vol. 34, no. 10, pp. 1305–1308, 2012.

[47] F. Dion, C. Dumayne, N. Henley et al., "Mechanism of insulin resistance in a rat model of kidney disease and the risk of developing type 2 diabetes," *PLoS One*, vol. 12, no. 5, article e0176650, 2017.

[48] P. Boor, P. Bozek, P. Blazícek et al., "Increased levels of circulating advanced glycation end products in a model of acute renal insufficiency in rats," *Casopís lékařů českých*, vol. 140, pp. 375–380, 2001.

[49] A. E. M. Stinghen, Z. A. Massy, H. Vlassara, G. E. Striker, and A. Boullier, "Uremic toxicity of advanced glycation end products in CKD," *Journal of the American Society of Nephrology*, vol. 27, no. 2, pp. 354–370, 2016.

[50] M. C. Thomas, C. Tsalamandris, R. Macisaac et al., "Low-molecular-weight AGEs are associated with GFR and anemia in patients with type 2 diabetes," *Kidney International*, vol. 66, no. 3, pp. 1167–1172, 2004.

[51] T. Miyata, C. van Ypersele de Strihou, K. Kurokawa, and J. W. Baynes, "Alterations in nonenzymatic biochemistry in uremia: origin and significance of "carbonyl stress" in long-term uremic complications," *Kidney International*, vol. 55, no. 2, pp. 389–399, 1999.

[52] K. Šsebeková, P. Blažíček, D. Syrová et al., "Circulating advanced glycation end product levels in rats rapidly increase with acute renal failure," *Kidney International*, vol. 59, pp. S58–S62, 2001.

[53] S. Gouroju, P. V. L. N. S. Rao, A. R. Bitla, K. S. Vinapamula, S. M. Manohar, and S. Vishnubhotla, "Role of gut-derived uremic toxins on oxidative stress and inflammation in patients with chronic kidney disease," *Indian Journal of Nephrology*, vol. 27, no. 5, pp. 359–364, 2017.

[54] P. A. Peterson, P. E. Evrin, and I. Berggård, "Differentiation of glomerular, tubular, and normal proteinuria: determinations of urinary excretion of β_2-macroglobulin, albumin, and total protein," *Journal of Clinical Investigation*, vol. 48, no. 7, pp. 1189–1198, 1969.

[55] M. Fox and M. F. A. Woodruff, "Effect of whole-body irradiation on the rate of increase of blood urea nitrogen following bilateral nephrectomy in rats and rabbits," *Nature*, vol. 202, no. 4935, pp. 918-919, 1964.

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Research Article

A Novel Heterozygous Mutation of the *COL4A3* Gene Causes a Peculiar Phenotype without Hematuria and Renal Function Impairment in a Chinese Family

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Mutations in the *COL4A3* gene are frequently reported to be associated with various types of hereditary nephropathy. *COL4A3* encodes the α3 chain of type IV collagen, which is the main structural protein in the basement membrane. Mutations in this gene are always related to kidney performance, and deafness and ocular lesion have also been reported. In this study, using next-generation sequencing, we investigated the DNA of a family visiting a clinic for hearing loss. A new missense mutation was found in *COL4A3* of 5 patients, c.3227C>T (p.P1076L). Based on these results, we predict that the mutation is pathogenic and leads to abnormal collagen IV. Here, we report for the first time on this autosomal dominant syndrome, characterized by hearing loss and eye abnormalities, but without renal damage, in all carriers. Since the oldest patient in the trial was less than 50 years old, however, we recommend that renal examination be reviewed regularly. Our results reveal expansion in the mutation spectrum of the *COL4A3* gene and phenotypic spectrum of collagen IV disease. Our study suggests that next-generation sequencing is an economical and effective method and may help in the accurate diagnosis and treatment of these patients.

1. Introduction

Type IV collagen, the major structural component of basement membranes (BMs), is a multimeric protein composed of 3 α subunits. These subunits are encoded by 6 different genes, α 1 through α 6, each of which can form a triple helix structure with 2 other subunits to form type IV collagen [1]. Trimers composed of α3, α4, and α5 chains are restricted in expression to specific BMs of the kidney, inner ear, and eye [2]. The *COL4A3* gene encodes α3 chains, and it is concluded that pathogenic *COL4A3* mutations account for Alport syndrome (AS) [3]. Variants in the *COL4A3* gene also cause diabetic kidney disease from maturity-onset diabetes in the young and those with familial benign hematuria (FBH) [4]. In addition, recent studies support that the *COL4A3* gene is a newly discovered pathogenic gene related to focal segmental glomerulosclerosis (FSGS) [5–7]. Autosomal dominant Alport syndrome causes hematuria, proteinuria, or progressive kidney disease. It also can be accompanied by late-onset high-tone sensorineural hearing loss and/or ocular abnormalities [8]. Hearing loss is directly related to the progression of renal failure in Alport syndrome [9]. The characteristics of FBH are persistent or recurrent hematuria without proteinuria, renal failure, or extra renal symptoms [10]. FSGS is a kind of proteinuria and end-stage renal disease (ESRD) associated with glomerular tissue pathological changes [11].

Recently, we enrolled a Chinese family on the clinic with a new mutation in the *COL4A3* gene. Profound hearing loss and retinitis pigmentosa (RP) were found in pathogenic gene carriers, but kidney impairment was not present. Mutations in the *COL4A3* gene that often lead to more atypical

phenotypes have received increasing attention, because clarifying the new pathogenesis can promote the development of new treatment methods and create new areas of research on hearing impairment. Targeted next-generation sequencing (NGS) was used to identify the genetic cause of deafness in this family.

2. Materials and Methods

2.1. Subjects. The program was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. The present study recruited all 7 family members. In addition, 250 healthy donors were recruited as controls for a mutation analysis. All participants involved in the study were of Han ethnicity and provided written informed consent prior to the study's commencement.

2.2. Phenotype Evaluation. The hearing levels of all participating members were measured by pure tone audiometry or auditory brainstem response. Ophthalmologic examination included fundus photography, a visual field test, and an electroretinogram. Through physical and radiological examination, pathological changes in the middle ear were excluded as were the presence of cochlear lesions and vestibular aqueduct syndrome. Additional auditory evaluations included otoscopic examination, an otoacoustic emissions test, and temporal bone high-resolution computed-tomography scanning. Examinations also included ophthalmologic exams, routine urine exam, urine protein quantitation, and blood biochemical analysis (retinal and liver function) of all family members.

2.3. Genetic Analysis. To make a precise diagnosis, we performed targeted NGS of related deafness genes. The collection of 5 mL peripheral blood samples taken from individuals was used for the following experiment. A capture panel (NimbleGen, Madison, USA) of deafness genes had been previously designed and assessed by our group. The capture panel included all exons together with the flanking exon and intron boundaries (±15 bp) of 180 genes. Genomic DNA of each subject was extracted using a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). The DNA was then fragmented by sonication using a Covaris LE220 (Woburn, Massachusetts, USA) to generate a paired-end library (200–250 bp). The library was enriched by array hybridization [12]. The magnitude of enrichment of the products was then evaluated with an Agilent 2100 Bioanalyzer (Santa Clara, California, USA) and ABI StepOne™ real-time PCR system (Applied Biosystems, Foster, CA, USA). Then, captured library sequencing was carried out using a BGISEQ-500 analyzer (BGI, Shenzhen, China) following the manufacturer's standard sequencing protocols to generate paired-end reads. Image processing and base calling were executed using Illumina Pipeline software (version 1.3.4) to generate raw data.

2.4. Mutational Analysis. We performed bioinformatics processing and analyzed the data after receiving the raw data. Previously published filtering criteria were used to generate "clean reads" for further analysis [12]. The "clean reads" (with a length of 90 bp) originated in targeted sequencing and filtering were then aligned to the human genome reference (hg19) using the BWA (Burrows-Wheeler Aligner) Multi-Vision software package [13]. We used SOAPsnp software [14] and SAMtools [15] to detect SNVs and indels, respectively. All SNVs and indels were filtered and estimated through cross-referencing against multiple databases, including NCBI dbSNP, HapMap, 1000 Genomes project, and a database containing DNA from 250 healthy Chinese adults. We used the following three different bioinformatics algorithms to predict the variants, including missense mutations and frame shift mutations: Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), and Mutation Master (http://www.Mutationtaster.org/). Furthermore, amino acid conservation was analyzed using the UniProt database (https://www.uniprot.org/).

2.5. Sanger Sequencing. Candidate gene mutations were validated using conventional Sanger sequencing methods. The primers were designed using the online Primer3 software. The PCR products were purified by shrimp alkaline enzyme (SAP) (from Promega) and exogenous enzyme I (EXO I) (from Epicentre) and sequenced using a BigDye® Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Foster, CA, USA). An ABI 3730xl sequencer (Carlsbad, CA, USA) was used to analyze the sequencing products. The sequencing documents were analyzed by PolyPhred software.

3. Results

3.1. Clinical Phenotype. Figure 1 shows the audiograms of family members. The proband and his sister had bilateral hearing loss that began in their first decade and gradually developed to severe hearing loss in the second decade. The proband's mother had mild hearing loss while his father was completely normal. No inner ear malformation of any subject was found by temporal bone high-resolution computed-tomography scanning. Ophthalmoscopy demonstrated retinal bone spicule pigmentary changes, retinal vessel attenuation, and diffuse macular atrophy in both eyes of the proband (Figure 2(a)). The proband and his sister also suffered from night vision disturbance and reduction of the visual field (Figure 2(b)). The proband and his sisters had an established clinical diagnosis of RP (Figure 2(c)). However, the proband's mother was unaffected by RP, but she was nyctalopic. But son (III-1) of II-3, a 2-year-old male, had normal phenotypes in the ear, eye, and kidney. All family members received routine blood and urine tests (including urinary sediment quantification), which showed no hematuria, proteinuria, or impaired renal function. Clinical characteristics are presented in Table 1 for all members.

3.2. Identification of Pathogenic Mutations in COL4A3. Sequence analysis of DNA in this pedigree identified a novel heterozygous mutation: C to T in exon 38 of *COL4A3* (NM_000091, c.3227C>T), which led to an amino acid substitution (p.Pro1076Leu) (Figure 3(a)). The missense mutation in *COL4A3* was absent in the 250 healthy controls. The

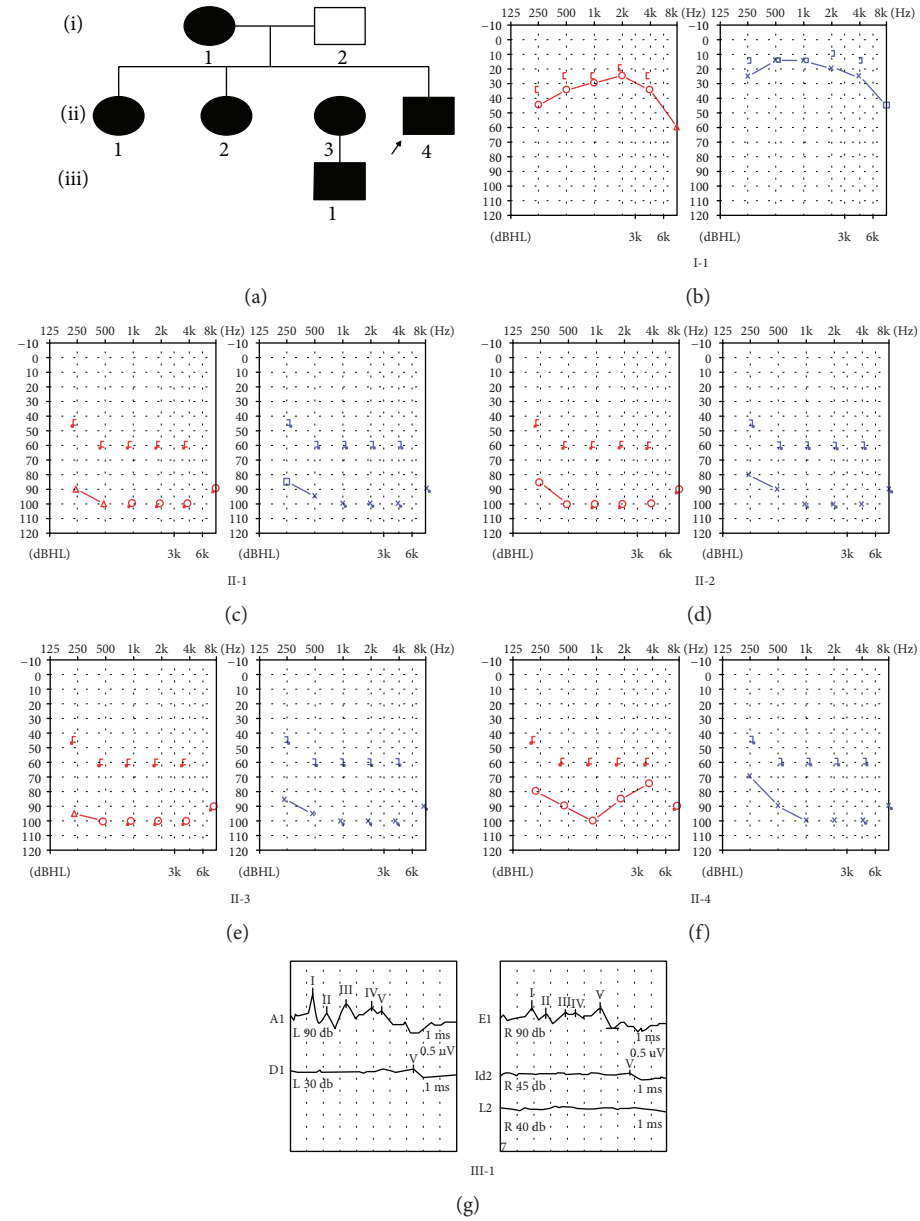

Figure 1: (a) Pedigree of a family with the COL4A3 mutation. Affected family members are shown in black symbols. The arrow identifies the proband in the family. (b–f) Audiogram of the affected members in the family. The red mark represents the right ear, and the blue mark represents the left ear. (g) Result of auditory brainstem response in III-1. The results show that the child's hearing is normal.

mutation was found in I-1, II-1, II-2, II-3, II-4, and III-1, consistent with an autosomal dominant mode of inheritance.

3.3. Bioinformatics Analysis of the COL4A3 Mutation. The variant sequence was highly conserved across different species (Figure 3(b)). To further evaluate the possible deleterious consequence of the novel COL4A3 missense mutation, three different in silico algorithms were used. SIFT analysis classified the variant as "intolerance" with a score of 0.01 (the threshold for intolerance is 0.05). The PolyPhen-2 software predicted the p.P1076L substitution to be probably damaging, with a score of 0.732. Additionally, the MutationTaster software supported the pathogenic effect of the variant, which was reported to be "disease-causing." These results suggest that the novel variant would substantially alter the function of the resulting protein.

4. Discussion

In this study, we identified a novel mutation (c.3227C>T) in *COL4A3*. Mutations in *COL4A3* always lead to kidney-related pathogenic phenotypes, but the family in the study presented recognized diagnostic challenges in kidney disease, including atypical clinical features. Increasing evidence has shown that identical variants in *COL4A3* are benign, but related to autosomally recessive AS, FSGS, and FBH [1, 5, 10]. The transmission of AS can be autosomal dominant, autosomal recessive, or X-linked, and

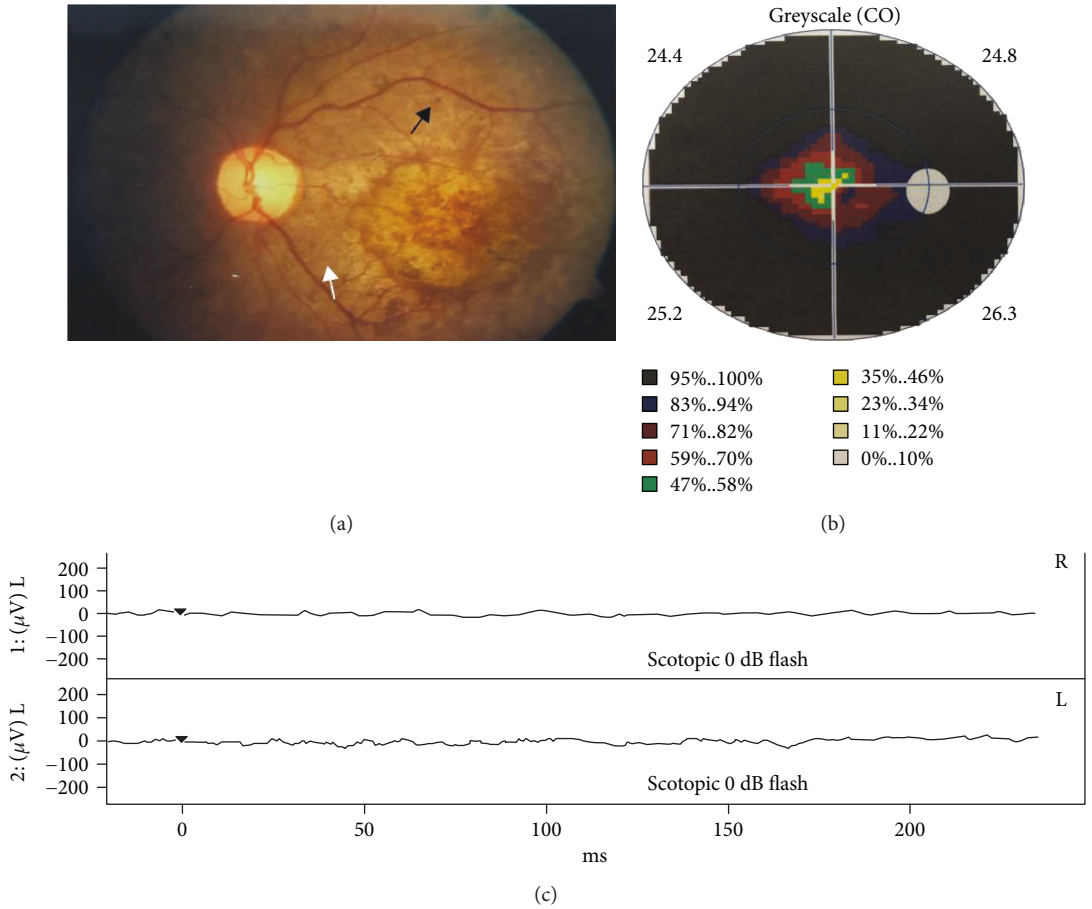

Figure 2: (a) Retina (right) reveals a retinopathy pigmented with attenuated retinal arterioles (white arrows). The midperiphery reveals bone spicule-like pigmentation (black arrows). (b) Reduction of the visual field. (c) An electroretinogram indicated dysfunction in the binocular retina.

Table 1: Clinical and genetic data for the family with the *COL4A3* c.3227C>T (p.P1076L) mutation.

Subject	I-1	I-2	II-1	II-2	II-3	II-4	III-1
Age	47	50	20	21	22	16	2
Gender	F	M	F	F	F	M	M
Hearing loss	Mild	Normal	Profound	Profound	Profound	Profound	Normal
Hematuria	No	No	No	No	No	No	No
Microhematuria	No	No	No	No	No	No	No
Proteinuria (g/24 h)	0.095	0.099	0.100	0.106	0.120	0.059	0.008
Creatinine (μmoI/L)	54	66	58	67	59	73	27
Renal failure	No	No	No	No	No	No	No
Nyctalopia	Yes	No	Yes	Yes	Yes	Yes	No
Visual field loss	No	No	Yes	Yes	Yes	Yes	No
Pigmented retinopathy	No	No	Yes	Yes	Yes	Yes	No
Genotype	Heterozygote	Normal	Heterozygote	Heterozygote	Heterozygote	Heterozygote	Heterozygote

M/F: male/female.

the prognosis is poor. On the contrary, BFH showed a good autosomal dominant pattern of prognosis [16]. All *COL4A3* mutations in previous research are summarized in Table 2. The age of onset is inconsistent. Most patients had the renal and extra renal phenotype, while others had only the renal phenotype. Rosado et al. found another *COL4A3* heterozygous mutation, which, in a few of the carriers, was also associated with a lack of kidney disease, but there was mild deafness [9]. However, a particular *COL4A3* mutation without renal damage in all carriers

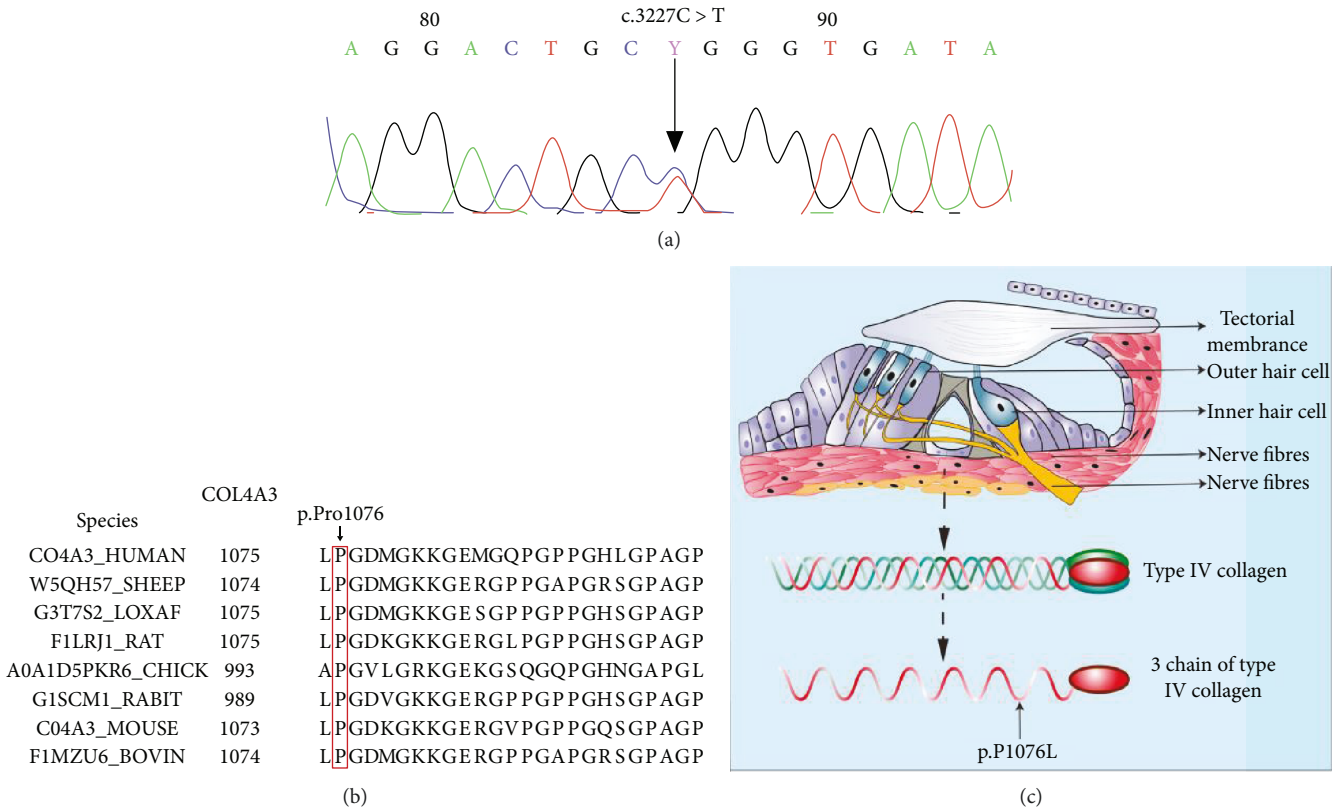

Figure 3: (a) Heterozygous mutation from C to T in exon 38 of COL4A3 identified by sequence analysis. (b) Conservation analysis of the collagen type IV α3 chain p.Pro1076 amino acid residue. The result shows that the amino acids at this site are highly conservative, and the mutant amino acids might have a great influence on structure and function. (c) Schematic diagram of the molecular structure of type IV collagen in the organ of Corti and amino acid mutation sites in the α3 chain of type IV collagen. Type IV collagen is abundantly expressed in the basement membrane, and mutation will affect the structure and function of the whole cochlea.

has not been reported. The proband and his sister in this study had severe hearing loss and eye abnormalities, but no renal impairments, such as hematuria and proteinuria, were found. In addition, the III-1, 2-year-old male, had no abnormality phenotype. We hoped that the ultrastructure of the glomerular basement membrane could be examined by renal biopsy, but this diagnostic test was refused.

Type IV collagen is usually an extracellular structural protein and constitutes a collagen branch network, which is an important component of BMs [1]. The type IV collagen molecule consists of three chains, each of which consists of three similar components: (1) a "7S" domain at the amino terminus, (2) a long collagen domain of about 1400 Gly-X-Y repeats interrupted by a short non-collagen region and forming a triple helix with the other two chains—the X and Y positions are usually proline and hydroxyproline but also can be any residue—and (3) a non-collagen "NC1" domain of about 230 residues at the carboxyl terminus, folded into a globular structure. *COL4A3* is located on 2q36-37, encoding an α3 collagen chain of type IV collagen [5, 27]. Figure 3 shows a schematic diagram of the molecular structure of type IV collagen in the organ of Corti and amino acid mutation sites of the α3 chain of type IV collagen in our study.

Even if environmental factors and different lifestyles influence the characteristics of disease in different family members [21], protein structure affected by mutation is the most important. The mechanism by which the mutation affects the function of *COL4A3* is not fully understood, yet the different clinical manifestations of the *COL4A3* mutation can be explained in this way. By integrating the altered collagen chain into the final network, normal structure and function are disrupted [28].

The *COL4A3* mutation in this family is located in the collagen domain. Proline can form an alpha helix structure in protein, destroying the α-helix, which will destroy the collagen skeleton. Type IV collagen α3, α4, and α5 chains are known to aggregate in the endoplasmic reticulum (ER) to form helical heterotrimers. Mutated *COL4A3* chains retained in the ER are associated with activation of the UPR pathway, leading to cytotoxicity and apoptosis [29]. Synthesis of the same amount of normal and abnormal chains will lead to the 1 : 1 ratio between the abnormal and normal molecules. Abnormal collagen homopolymers may cause the molecular folding, secretion, and extracellular matrix formation [30]. Other similar studies have also mentioned that misfolded proteins can be secreted into BM or accumulate in podocytes, disrupting glomerular selective barrier properties and activating downstream pathological pathways [31, 32]. Zehnder et al. discovered that in human cochlea, the α3 chain is specifically expressed in collagen bundles in the basement membrane, spiral ligament, and spiral margin. These results

Table 2: The clinical characteristics of patients carrying *COL4A3* mutations.

References	Populations	Age	Gender	Mutation state	Mutation	Hematuria	Proteinuria	Hearing loss	Ocular lesions
[16]	UK	47	M	Heterozygous	c.3418+1G>T c.4664C>T;	+	+	+	+
[17]	Spanish	58	M	Heterozygous	c.998G>A;	+	+	+	—
[18]	Sri Lankan	14	F	Homozygous	c.1219G>T; c.1223_ 1224delGG	+	+	+	—
[9]	Spanish	32	F	Heterozygous	c.345 delG;	+	+	+	+
[19]	Ashkenazi Jewish	2	F	Homozygous	c.40_63del	+	+	+	—
[19]	UK	14	UK	Heterozygous	c.40_63del	+	+	—	—
[19]	Spanish	UK	F	Compound heterozygote	c.40_63del	—	+	+	+
[20]	Cypriot	32	F	Heterozygosity	c.2621-2622delGAinsT	+	+	+	+
[20]	Cypriot	UK	M	Heterozygosity	c.3229G>A	+	+	—	—
[6]	Caucasian	8	F	Compound heterozygous	del393G_E131fsX151 2806C>T	+	+	+	—
[6]	Caucasian	35	F	Heterozygous	c443G>T	+	+	—	—
[6]	Caucasian	33	M	Heterozygous	c4981C>T	_	+	—	—
[6]	Caucasian	36	F	Heterozygous	c2083G>A	+	+	+	—
[21]	Chinese	45	M	Heterozygous	c. 2290G >A	+	+	—	—
[22]	Turkish	15	F	Heterozygous	C.2T>C	+	+	+	+
[23]	Italian	13	F	Heterozygous	c.872G>A	+	+	—	—
[24]	African American	14	M	Compound heterozygous	c.4486C>T c.4546C>T	+	+	+	—
[25]	Slovenia	56	F	Heterozygous	c.3547_3548in-sGGA	+	—	—	—
[25]	Slovenia	38	M	Heterozygous	c.1459G>T	+	—	—	—
[26]	Chinese	28	F	Homozygous	c.3725G>A	+	+	+	+
Current study	Chinese	16	M	Heterozygous	c.3227C>T	—	—	+	+
Current study	Chinese	47	F	Heterozygous	c.3227C>T	—	—	+	+
Current study	Chinese	2	M	Heterozygous	c.3227C>T	—	—	—	—

UK: unknown; M/F: male/female; +/-: positive/negative.

suggest that hearing loss may be due to cochlear micromechanical changes or are consistent with the helical ligament dysfunction hypothesis [2]. Type IV collagen $\alpha 3$ chains are also found in the basement membranes of the conjunctiva, cornea, iris, lens capsule, and Descemet's and Bruch's membrane [33]. Therefore, mutation in the COL4A3 gene leads to structural and functional disorders of type 4 collagen in the affected basement membrane. Ultimately, it leads to abnormal ocular phenotypes.

The special phenotype of *COL4A3* in this study may also be due to haploinsufficiency [34]. Studies have shown that the heterozygous *COL4A3* mutation can lead to a decrease in expression [4]. A mutant gene in an allele causes the amount of protein encoded by a heterozygous mutant gene to fail to reach the threshold required for the normal functioning of the protein encoded by the two alleles thus causing the protein encoded by the heterozygous mutant gene to fail to perform its normal physiological function, which is functionally equivalent to a nonsense mutation [35]. If haploinsufficiency is a mechanism by which *COL4A3* mutations affect gene expression regulation, the kidney can avoid the effect of partial loss of *COL4A3* function, because COL4A3 may be expressed at a much higher level in the kidney than in the cochlea and eye [36, 37]. Thus, COL4A3 levels may be sufficient to maintain kidney function, which may not be the case in the cochlea and eyes that exhibit much lower levels of expression. Alternatively, the mutant COL4A3 protein may lose some of its functions, but not others, rather than just lowering the overall level of COL4A3 function. If the key function is related to the cochlea and the eye, but not to the kidney, the mutation can affect the cochlea and the eye, but not the kidney.

One of the important specificities of the heterozygous *COL4A3* mutation reported here is that the renal phenotype was not found in I-1, II-1–II-4, and III-1. The wide phenotypic transformation with *COL4A3* mutations and the

presence of incomplete penetrance suggest that a simple Mendelian model is inadequate to explain the genetic mechanism of the disease [38].

5. Conclusion

In summary, the study expanded the phenotypic spectrum of *COL4A3* mutation carriers. It is noteworthy that although we have found a heterozygous *COL4A3* mutation (c.3227 C>T) that may cause extreme deafness and ocular abnormalities, we should continue to monitor possible delayed kidney disease. Further, the autosomal dominant pattern in this case suggests to us the importance of this mode to deafness and ocular abnormalities, though 70 to 85% of the mode of inheritance of neurosensory hearing impairment occurs in an autosomal recessive pattern. Moreover, the novel pathogenic mutation identified in this study will be important for carrier testing and premarital screening for this family and their relatives in the future.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no competing interest relevant to the publication of this paper.

Acknowledgments

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References

[1] L. Heidet, C. Arrondel, L. Forestier et al., "Structure of the human type IV collagen gene *COL4A3* and mutations in autosomal Alport syndrome," *Journal of the American Society of Nephrology*, vol. 12, no. 1, pp. 97–106, 2001.

[2] A. F. Zehnder, J. C. Adams, P. A. Santi et al., "Distribution of type IV collagen in the cochlea in Alport syndrome," *Archives of Otolaryngology – Head & Neck Surgery*, vol. 131, no. 11, pp. 1007–1013, 2005.

[3] H. H. Lemmink, T. MochlzukJ, L. P. W. J. van den Heuvel et al., "Mutations in the type IV collagen α3 (COL4A3) gene in autosomal recessive Alport syndrome," *Human Molecular Genetics*, vol. 3, no. 8, pp. 1269–1273, 1994.

[4] Y. Wang, J. Zhang, Y. Zhao et al., "*COL4A3* gene variants and diabetic kidney disease in MODY," *Clinical Journal of the American Society of Nephrology*, vol. 13, no. 8, pp. 1162–1171, 2018.

[5] X. Jingyuan, W. Xiaoxi, R. Hong et al., "COL4A3 mutations cause focal segmental glomerulosclerosis," *Journal of Molecular Cell Biology*, vol. 6, no. 6, pp. 498–505, 2014.

[6] A. F. Malone, P. J. Phelan, G. Hall et al., "Rare hereditary COL4A3/COL4A4 variants may be mistaken for familial focal segmental glomerulosclerosis," *Kidney International*, vol. 86, no. 6, pp. 1253–1259, 2014.

[7] K. Voskarides, A. Pierides, and C. Deltas, "*COL4A3*/*COL4A4* mutations link familial hematuria and focal segmental glomerulosclerosis. Glomerular epithelium destruction via basement membrane thinning?," *Connective Tissue Research*, vol. 49, no. 3-4, pp. 283–288, 2009.

[8] F. D. L. Van, L. Heidet, E. D. Timmer et al., "Autosomal dominant Alport syndrome caused by a COL4A3 splice site mutation," *Kidney International*, vol. 58, no. 5, pp. 1870–1875, 2000.

[9] C. Rosado, E. Bueno, P. Fraile, P. García-Cosmes, and R. González-Sarmiento, "A new mutation in the COL4A3 gene responsible for autosomal dominant Alport syndrome, which only generates hearing loss in some carriers," *European Journal of Medical Genetics*, vol. 58, no. 1, pp. 35–38, 2015.

[10] C. Badenas, M. Praga, B. Tazón et al., "Mutations in the *COL4A4* and COL4A3 genes cause familial benign hematuria," *Journal of the American Society of Nephrology*, vol. 13, no. 5, pp. 1248–1254, 2002.

[11] Y. Wu, P. Hu, H. Xu et al., "A novel heterozygous *COL4A4* missense mutation in a Chinese family with focal segmental glomerulosclerosis," *Journal of Cellular and Molecular Medicine*, vol. 20, no. 12, pp. 2328–2332, 2016.

[12] X. Wei, X. Ju, X. Yi et al., "Identification of sequence variants in genetic disease genes using targeted next-generation sequencing," *PLoS One*, vol. 6, no. 12, article e29500, 2011.

[13] H. Li and R. Durbin, "Fast and accurate short read alignment with Burrows–Wheeler transform," vol. 25, no. 14, pp. 1754–1760, 2009.

[14] R. Li, Y. Li, X. Fang et al., "SNP detection for massively parallel whole-genome resequencing," *Genome Research*, vol. 19, no. 6, pp. 1124–1132, 2009.

[15] H. Li, B. Handsaker, A. Wysoker et al., "The sequence alignment/map format and SAMtools," *Bioinformatics*, vol. 25, no. 16, pp. 2078-2079, 2009.

[16] A. Li, Y. X. Cui, X. Lv et al., "The *COL4A3* and *COL4A4* digenic mutations in cis result in benign familial hematuria in a large Chinese family," *Cytogenetic and Genome Research*, vol. 154, no. 3, pp. 132–136, 2018.

[17] C. Cerveraacedo, A. Coloma, E. Huarteloza, M. Sierracarpio, and E. Domínguezgarrido, "Phenotype variability in a large Spanish family with Alport syndrome associated with novel mutations in *COL4A3* gene," *BMC Nephrology*, vol. 18, no. 1, p. 325, 2017.

[18] N. D. Sirisena, S. Thalgahagoda, A. Abeyagunawardena et al., "Novel *COL4A3* gene mutations in a consanguineous family with autosomal recessive Alport syndrome," *Nephrology*, vol. 20, no. 8, p. 580, 2015.

[19] B. D. Webb, T. Brandt, L. Liu et al., "A founder mutation in *COL4A3* causes autosomal recessive Alport syndrome in the Ashkenazi Jewish population," *Clinical Genetics*, vol. 86, no. 2, pp. 155–160, 2014.

[20] L. Papazachariou, P. Demosthenous, M. Pieri et al., "Frequency of *COL4A3*/*COL4A4* mutations amongst families segregating glomerular microscopic hematuria and evidence for activation of the unfolded protein response. Focal and segmental glomerulosclerosis is a frequent development during ageing," *PLoS One*, vol. 9, no. 12, article e115015, 2014.

[21] F. Lin, B. Fan, J. Zou et al., "Whole exome sequencing reveals novel *COL4A3* and *COL4A4* mutations and resolves diagnosis in Chinese families with kidney disease," *BMC Nephrology*, vol. 15, no. 1, p. 175, 2014.

[22] A. S. Uzak, B. Tokgoz, M. Dundar, and M. Tekin, "A novel *COL4A3* mutation causes autosomal-recessive Alport syndrome in a large Turkish family," *Genetic Testing and Molecular Biomarkers*, vol. 17, no. 3, pp. 260–264, 2013.

[23] J. Hoefele, B. Lange-Sperandio, D. Ruessmann et al., "Novel heterozygous COL4A3 mutation in a family with late-onset ESRD," *Pediatric Nephrology*, vol. 25, no. 8, pp. 1539–1542, 2010.

[24] C. Cook, C. A. Friedrich, and R. Baliga, "Novel *COL4A3* mutations in African American siblings with autosomal recessive Alport syndrome," *American Journal of Kidney Diseases*, vol. 51, no. 5, pp. e25–e28, 2008.

[25] M. Slajpah, B. Gorinsek, G. Berginc et al., "Sixteen novel mutations identified in COL4A3, COL4A4, and COL4A5 genes in Slovenian families with Alport syndrome and benign familial hematuria," *Kidney International*, vol. 71, no. 12, pp. 1287–1295, 2007.

[26] P. Hou, Y. Chen, J. Ding, G. Li, and H. Zhang, "A novel mutation of *COL4A3* presents a different contribution to Alport syndrome and thin basement membrane nephropathy," *American Journal of Nephrology*, vol. 27, no. 5, pp. 538–544, 2007.

[27] H. Storey, J. Savige, V. Sivakumar, S. Abbs, and F. A. Flinter, "COL4A3/COL4A4 mutations and features in individuals with autosomal recessive Alport syndrome," *Journal of the American Society of Nephrology*, vol. 24, no. 12, pp. 1945–1954, 2013.

[28] D. J. Tilstra and P. H. Byers, "Molecular basis of hereditary disorders of connective tissue," *Annual Review of Medicine*, vol. 45, no. 1, pp. 149–163, 1994.

[29] H. Yoshida, "ER stress and diseases," *FEBS Journal*, vol. 274, no. 3, pp. 630–658, 2007.

[30] U. Schwarze, J. A. Goldstein, and P. H. Byers, "Splicing defects in the COL3A1 gene: marked preference for 5′ (donor) splice-site mutations in patients with exon-skipping mutations and Ehlers-Danlos syndrome type IV," *American Journal of Human Genetics*, vol. 61, no. 6, pp. 1276–1286, 1997.

[31] D. R. Abrahamson, B. G. Hudson, L. Stroganova, D. B. Borza, and P. L. St John, "Cellular origins of type IV collagen networks in developing glomeruli," *Journal of the American Society of Nephrology*, vol. 20, no. 7, pp. 1471–1479, 2009.

[32] M. Pieri, C. Stefanou, A. Zaravinos et al., "Evidence for activation of the unfolded protein response in collagen IV nephropathies," *Journal of the American Society of Nephrology*, vol. 25, no. 2, pp. 260–275, 2014.

[33] D. Colville, J. Savige, M. Morfis et al., "Ocular manifestations of autosomal recessive Alport syndrome," *Ophthalmic Genetics*, vol. 18, no. 3, pp. 119–128, 2009.

[34] W. J. Song, M. G. Sullivan, R. D. Legare et al., "Haploinsufficiency of *CBFA2* causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia," *Nature Genetics*, vol. 23, no. 2, pp. 166–175, 1999.

[35] A. Sánchez-Mejías, Y. Watanabe, R. M. Fernández et al., "Involvement of SOX10 in the pathogenesis of Hirschsprung disease: report of a truncating mutation in an isolated patient," *Journal of Molecular Medicine*, vol. 88, no. 5, pp. 507–514, 2010.

[36] A. Leinonen, "Complete primary structure of human α4 (IV) collagen chain," *Journal of Biological Chemistry*, vol. 269, 1994.

[37] S. Y. Nishio, Y. Takumi, and S. I. Usami, "Laser-capture micro dissection combined with next-generation sequencing analysis of cell type-specific deafness gene expression in the mouse cochlea," *Hearing Research*, vol. 348, pp. 87–97, 2017.

[38] M. A. Mencarelli, L. Heidet, H. Storey et al., "Evidence of digenic inheritance in Alport syndrome," *Journal of Medical Genetics*, vol. 52, no. 3, pp. 163–174, 2015.