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Diagnosis, prevention and treatment in diabetic nephropathy - volume II

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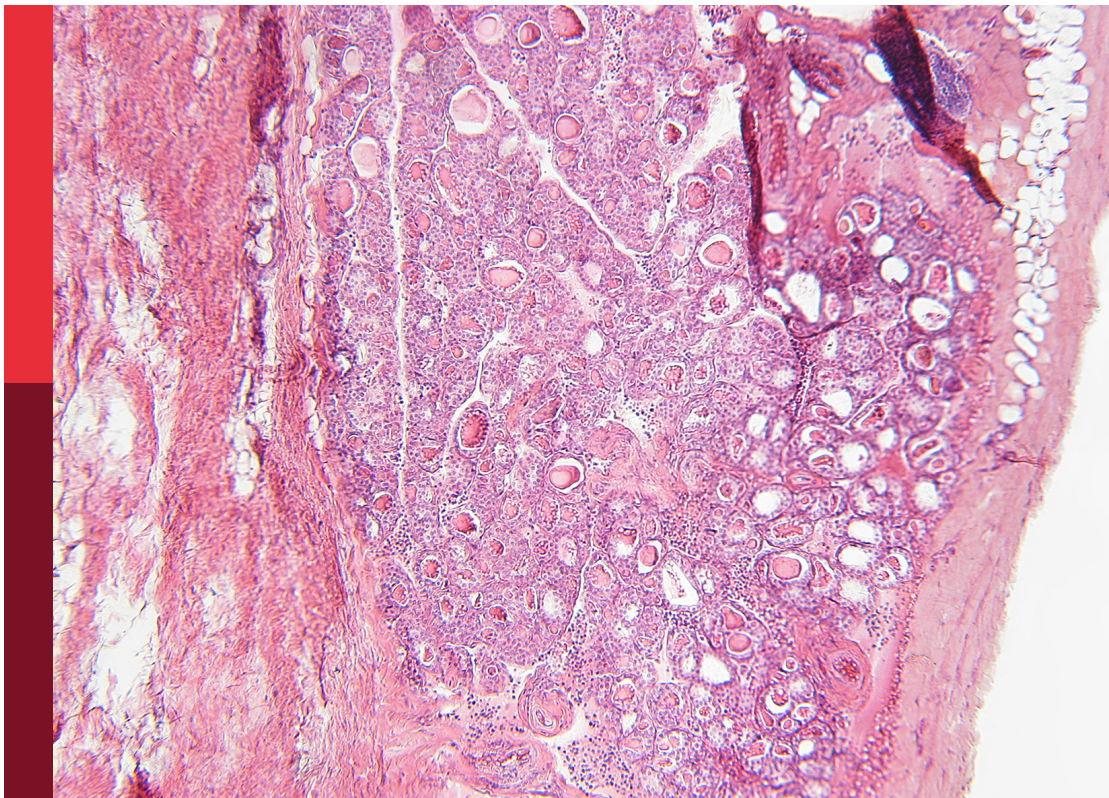

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Diagnosis, prevention and treatment in diabetic nephropathy - volume II

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Editorial: Diagnosis, prevention and treatment in diabetic nephropathy, volume II

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Editorial on the Research Topic
Diagnosis, prevention and treatment in diabetic nephropathy, volume II

The number of diabetic patients worldwide has more than tripled in the past two decades, and approximately one-third of people with diabetes mellitus (DM) eventually develop diabetic kidney disease (DKD), of which approximately 50% may progress to end-stage disease (ESRD). DKD is the most relevant microvascular complication of diabetes together with retinopathy and neuropathy. ESRD is associated with a high cardiovascular mortality rate, risk of hospitalization, and all-cause mortality in patients with diabetes and places a huge economic burden on patients and society. In addition, ESRD harms patients' psychological status due to disabling morbidity and a large amount of disability-adjusted life years (DALYs).

While the global spread of DKD and its consequences are certain, a full understanding of the pathophysiology, diagnosis, and treatment of this condition is still lacking.

So far, a combination of inflammation and insulin resistance was thought to be responsible for the development of DKD and its cardiovascular (CV) complications, including death. Furthermore, clinical assessment and traditional biomarkers (such as eGFR and proteinuria) have been considered convenient tools to suspect DKD and monitor CKD progression. While pathological sample analysis has remained the gold standard method for diagnosis. The introduction of a new panel of biomarkers into clinical practice has been hampered by the resulting poor accuracy and specificity achieved until now. Finally, comprehensive management of DKD-promoting risk factors has been considered the cornerstone recommendation of current CKD treatment guidelines in DM.

However, recently an increasing body of evidence is showing critical gaps and open questions about this interesting Research Topic, and these new findings could change our current certainties, particularly regarding the diagnosis and treatment of DKD. Nearly 50% of patients with type 2 DM in stage 3 chronic kidney disease (CKD) remain undiagnosed. Traditional biomarkers, considered solid tools for diagnosis, are lacking in terms of sensitivity and specificity and the limitations of their therapeutic and prognostic value significantly condition their role in clinical practice. Additionally, the renal puncture is not widely available due to its invasiveness. Furthermore, even when DKD is diagnosed, there is still no effective therapy, and the only treatment options for late-stage DKD include dialysis or

kidney transplantation, which are expensive and significantly increase personal and social burdens. Moreover, DKD displays such clinical heterogeneity that it increases the difficulty of treatment, and even considerable effort to control risk factors and manage blood glucose has limited efficacy in preventing DKD progression. Therefore, there is an urgent need for sensitive diagnostic tools to successfully identify people at risk for DKD, and effective preventive and therapeutic strategies to improve patient outcomes.

Fortunately, recent technological advancement and cost reductions for modern diagnostic tests can promisingly help clinicians obtain a timely diagnosis of DKD that can delay its progression to ESRD. Furthermore, new insights into the pathogenic mechanisms of DKD could provide more possible directions for new therapies for DKD.

A deep understanding of the pathogenic process behind the development of DKD could open up new disease-modifying therapies, and remarkable discoveries have recently emerged.

Prebiotics, probiotics, diet, and antibiotic use influence microbial composition. However, also DKD promotes dysbiosis, which has been shown to contribute to renal disease progression by activating intrarenal RAS, promoting inflammation and fibrosis through increased TMAO production, and worsening tubulointerstitial damage through regulation of cholesterol homeostasis. Han et al. were the first to comprehensively characterize the different bacterial compositions in DKD compared to non-DKD subjects. In particular, an enrichment of Hungatella and Escherichia genera and depletion of butyrate-producing bacteria was observed. This could be a new revealing pathogenic condition associated with developing DKD.

DKD promotes impairment of mechanisms regulating oxidative stress and Wu and Chen observed an increase in intracellular iron accumulation, glutathione depletion, and lipid peroxidation in patients with CKD and DM. This phenomenon is called ferroptosis and is defined as iron-dependent regulated cell death, which involves the regulation of genes and proteins.

UA is an independent, modifiable risk factor for chronic kidney disease and a cause of CV and non-CV complications in DKD. Huang et al. found that this "old new biomarker" can also accurately reflect abnormal glomerular and/or tubular function since approximately 90% of its excretion is due to the kidneys.

Lastly, lipid metabolism is known to cause CVD, but Lu et al. showed how new lipid biomarkers and lipid indices may be associated with an increased risk of DKD.

Diagnosis is an open challenge for DKD. An accurate estimation of the glomerular filtration rate is critical for diagnosis and is also crucial for the classification and management of patients with CKD. However, measuring glomerular filtration rate using clearance of inulin, technetium-99m-diethylene triamine pentaacetic acid, iohexol, or 125I-iothalamate is invasive, inconvenient, and expensive to use in daily practice.

Estimation of eGFR using equations (including those recommended in guidelines such as the CKD-EPIcr-cys equation) has limitations regarding accuracy in older adults and has shown significantly lower accuracy in individuals with diabetes than in the non-diabetic group. Specifically, Jiang et al. observed that CKD-EPIcr and CKD-EPIcys overestimated and underestimated GFR, respectively.

In recent years, technological progress has reduced the costs of highly innovative diagnostic techniques. For example, whole genome transcriptome analysis has been used extensively in the field of DKD and could prove crucial in identifying a simplified panel of serum biomarkers that can predict the risk of developing DKD. Indeed, Wang et al. achieved high diagnostic efficacy with the combination of two markers. The expression of REG1A and RUNX3 was significantly increased in blood samples from DKD patients and may be a novel predictor of renal disease in DM.

Furthermore, proteomics-based analysis facilitated the identification of novel target proteins suitable for DKD diagnosis and progression. In particular, Huang et. al through pressure circulation technology and pulseDIA proteomic analysis identified additional sensitive markers for early detection of kidney disease from the blood and urine of diabetic patients with CKD. Specifically, the authors found that autophagy-related protein NBR1 was significantly upregulated in early and advanced DKD, while ATG4B and VPS37A were significantly downregulated with the progression of DKD.

Also traditional well-known biomarkers have also shown a promising new role in diagnosis. Lu et al. found that apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B), and lipid ratios may be associated with the onset of DKD to support diagnosis using readily available, noninvasive biomarkers.

At present, treatment options for DKD are limited and progression to ESRD appears inevitable. However, new findings on the pathogenesis of DKD could lead to promising new therapies. For instance, Wu and Chen noted that treating the processes underlying ferroptosis by modulating intracellular signaling pathways or using iron-chelating agents could be a new treatment option to slow the progression of DKD.

Lin et al. studied extracellular vesicles (EVs). They observed their beneficial effects on kidney injury stemming from their anti-apoptotic, anti-inflammatory, antioxidant, and anti-fibrotic role and their ability to modulate podocyte autophagy.

Furthermore, statins appear to exert some sort of beneficial effect on kidney damage by reducing proteinuria along with their ability to protect diabetic patients from cardiovascular events. However, they fail to delay the progression of DKD. Thus, novel therapies targeting novel lipid biomarkers could be a potent treatment for DKD(4).

Finally, promising new ways to monitor DKD progression and stratify individual risk can significantly change patient outcomes.

Shi et al. found that urinary IL-18 is associated with impaired carotid-femoral pulse wave velocity (cf-PWV), which is the current clinical gold standard measure of arterial stiffness and established cardiovascular risk marker in patients with T2D with DKD.

Interestingly, DKD affects body composition causing a gradual quantitative and qualitative deterioration of muscle mass. Therefore, sarcopenia is a common complication of DKD and worsens patient outcomes. Lin et al. observed a significant association between decreased rectus femoris cross-sectional area/increased visceral fat area and DKD progression, resulting in a promising and easily obtainable biomarker of prognosis.

Nutrition is confirmed to be one of the most relevant aspects to be investigated in our patients with chronic renal insufficiency. Indeed, as noted by Duan et al., T2DM patients with a reduced 25(OH) vitamin D level had worse renal function with an increased risk of DKD progression.

Finally, Huang et al. developed a new risk model that includes traditional risk factors that contribute to the early identification and prevention of complications in patients with DKD.

Overall, the published articles contribute to enriching current knowledge on the pathogenesis of DKD and the authors' innovative findings could answer the open questions regarding the need for sensitive diagnostic tools and effective therapeutic strategies. It is hoped that this is a promising step towards significantly improving the prognosis and outcomes of patients with DKD.

Author contributions

ALC, MMR, FB and AF wrote the article. FB and MMR participated as guest editors for manuscripts of the Research Topic, where they were not coauthors themselves. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Association of Serum 25 (OH) Vitamin D With Chronic Kidney Disease Progression in Type 2 Diabetes

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Objectives: Growing evidence demonstrated that vitamin D levels had been linked to type 2 diabetes mellitus (T2DM) and chronic kidney disease (CKD) in light of various extraskeletal effects. Therefore, the present study aimed to evaluate the association of 25-hydroxyvitamin D [25(OH)D] level with the clinicopathological features and CKD progression in T2DM.

Methods: A total of 182 patients with T2DM with CKD stages 1 through 4 (G1–G4) were retrospectively included. Identification of the serum 25(OH)D level associated with CKD progression was executed by Kaplan–Meier survival analysis and Cox proportional hazards models. We further performed sensitivity analyses with a time-weighted average (TWA) of the serum 25(OH)D level in 75 participants to reinforce the findings.

Results: The median serum 25(OH)D level was 26 (IQR, 14; 39) nmol/L in the study participants. Median follow-up time was 42 months, during which 70 (38%) patients confronted CKD progression. Cumulative kidney outcomes were significantly higher in the lowest tertile of the serum 25(OH)D level in Kaplan–Meier analyses ($P < 0.001$). Consistently, the analyses of Cox proportional hazards regression models indicated a significantly greater risk for CKD progression in the lowest tertile of the serum 25(OH)D level compared with the highest tertile of the serum 25(OH)D level ($P = 0.03$). These relationships remained robust with further sensitivity analysis of data with TWA of the serum 25(OH)D level, showing an independent association between lower TWA of the serum 25(OH)D level and an unfavorable renal outcome in patients with T2DM with CKD.

Conclusions: Our findings demonstrated that patients with T2DM with a decreased 25 (OH)D level had deteriorated renal function. Both lower levels of baseline and TWA of serum 25(OH)D were associated with an increased risk of CKD progression in patients with T2DM, which suggested that the long-term maintenance of optimal vitamin D levels from early in life might be associated with reduced future risk of CKD development in T2DM.

Keywords: 25-hydroxyvitamin D, type 2 diabetes mellitus, CKD progression, diabetic kidney disease, non-diabetic kidney disease

INTRODUCTION

The increasing prevalence of diabetes and its major microvascular complication, chronic kidney disease (CKD), has emerged globally as a substantial public health burden (1). The 10th edition of the International Diabetes Federation Atlas estimates that diabetes affected 537 million people in 2021 and is expected to reach 783 million by 2045, with the majority being type 2 diabetes mellitus (T2DM). Current data from the United States suggest that 37% of patients with diabetes were in coexistence with CKD stages 1 through 4 (G1–G4) and that 38% of end-stage kidney disease (ESKD) cases were on account of diabetes (2). In China, diabetes has been the primary cause of CKD since 2011, with an estimated 24.3 million diabetic patients living with CKD (3). Although significant progress has been made in the past three decades in the comprehensive treatment strategy, patients with T2DM remain at continuing high risk for progression of CKD, which is associated with increased cardiovascular complications, morbidity, and mortality (1, 2, 4). Individuals with T2DM plus superimposed CKD have been associated with approximately a three-fold increased risk of cardiovascular disease (CVD) and death than those with T2DM alone (5). Therefore, there is a compelling need to discover potential clinical indicators or prognostic factors to identify individuals at risk of CKD progression in T2DM who may benefit from early diagnosis and timely risk intervention in clinical practice.

As a pleiotropic steroid hormone, Vitamin D (VD) can exert various effects through binding to VD receptors (6). The primary function of VD is the regulation of calcium and phosphorus homeostasis to ensure adequate mineralization and bone growth (7). In addition, it plays a vital role in modulating cell proliferation, apoptosis, differentiation, inflammation response, immune function, and vascular and metabolic properties such as insulin secretion and insulin sensitivity (8–10). 25-*hydroxyvitamin D [25(OH)D] is synthesized by 25*-hydroxylase catalyzing VD, which is considered as the best indicator of VD status (11, 12). In recent years, lower serum 25(OH)D level has been implicated in the incidence of T2DM, which may rely on its association with impaired glucose and insulin metabolism (12, 13). More importantly, VD deficiency increases the risk of T2DM development and the incidence of its complication (14, 15). In particular, the prevalence of VD deficiency is very high in patients with CKD and the survival rates could be enhanced by active VD treatment (6, 16). A previous study reported that low 25(OH)D levels were associated with the development of ESKD (17). Nevertheless, it has not been fully elucidated about the relationship of the serum 25(OH)D level with kidney clinicopathologic features and renal outcomes in patients with T2DM. Furthermore, no literature has ever addressed the association between the time-weighted average (TWA) serum 25(OH)D level and CKD progression in patients with T2DM.

Hence, the current study set out to evaluate the significance of the serum 25(OH)D level for clinicopathological features and kidney progression, which was defined as a double increase in serum creatinine (D-Scr) from baseline values or occurrence of ESKD in T2DM with CKD. Further, to reinforce the point, the prospective association of TWA of the serum 25(OH)D level with the risk of CKD progression in T2DM was also investigated.

MATERIALS AND METHODS

Subjects

A total of 254 patients with diabetes mellitus (T2DM) with kidney diseases from January 2011 to December 2020 at the renal department of The First Affiliated Hospital of Nanjing Medical University were retrospectively reviewed. Finally, 182 patients were included and categorized into two groups: 141 patients with biopsy-proven diabetic kidney disease (DKD) and 41 patients with biopsy-proven non-DKD (NDKD) (**Figure 1**). T2DM was diagnosed according to the American Diabetes Association (18). The inclusion criteria were all patients diagnosed with T2DM complicated with CKD, defined as abnormalities of kidney structure or function, present for ≥3 months by the Kidney Disease:Improving Global Outcomes (KDIGO) Clinical Practice Guidelines. They had undergone renal biopsy pathological examination after excluding contraindications (19, 20). In addition, they should have intact information on the baseline serum 25(OH)D level. Exclusion criteria were as follows (1): advanced heart failure [the New York Heart Association (NYHA) functional classification III or IV]; (2) cirrhosis; (3) polycystic kidney disease; (4) other types of DM; (5) malignancies; (6) women with pregnancy; (7) acute inflammation or infections; (8) the estimated glomerular filtration rate (eGFR) ≤15 ml/min/1.73 m^2; and (9) patients with new-onset diabetes after transplantation and those who underwent renal replacement therapy before the biopsy. This study was approved by the Ethics Committee of The First Affiliated Hospital of Nanjing Medical University.

Clinical and Laboratory Parameters

The complete clinical and laboratory information of enrolled patients was collected at the time of renal biopsy, including age, gender, blood pressure, duration of diabetes, diabetic retinopathy, hypertension, body mass index (BMI), eGFR, blood urea nitrogen (BUN), serum creatinine (Scr), alkaline phosphatase (ALP), 25 (OH)D, serum albumin, serum calcium and phosphorus, fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), parathyroid hormone (PTH), 24-h urinary calcium and phosphorus excretion, serum immunoglobulin A (IgA), serum immunoglobulin G (IgG), complement C3, complement C4, serum and urinary neutrophil gelatinase-associated lipocalin (NAGL), urinary N-acetyl-β-D glucosaminidase (uNAG), and retinol-binding protein (RBP).

Serum 25(OH)D measurements in the follow-up period were averaged into TWA serum 25(OH)D level for each patient. The TWA value was derived as an aggregate area under the curve divided by the cumulative time exposure for each patient. The area under the curve was measured as an integrated expression over time using a positive incremental

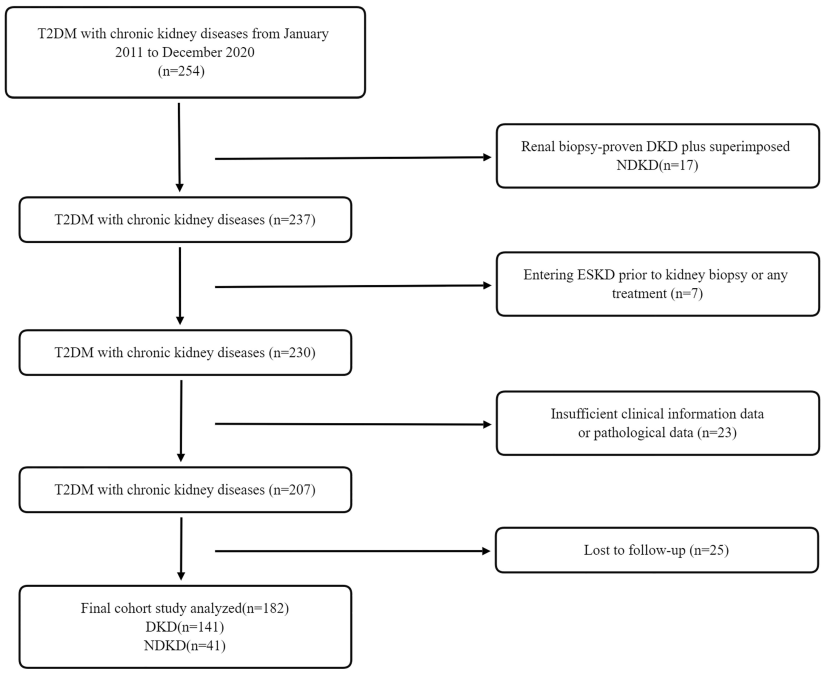

FIGURE 1 | Flowchart of study participants. T2DM, type 2 diabetes mellitus; DKD, diabetic kidney disease; NDKD, non-diabetic kidney disease; CKD, chronic kidney disease; ESKD, end-stage kidney disease.

method, without imputation for missing time points. The calculated formula is as follows: TWA serum 25(OH)D = $\{[(X_1 + X_2)(T_2 - T_1) + (X_2 + X_3)(T_3 - T_2) + \ldots + (X_{n-1} + X_n)(T_n - T_{n-1})]/[2\times(T_n - T_1)]\}$, where T_n is nth time point and X_n is the serum 25(OH)D level at T_n (21).

The current medications of participants were also recorded, including blood pressure–lowering therapy [renin-angiotensin-aldosterone system inhibitor (RAASi), β-blocker, diuretic and calcium-channel blocker (CCB), statins, insulin, and oral hypoglycemic agents]. The serum 25(OH)D level was determined by electrochemiluminescence immunoassay (Roche Diagnostic GmBH, Germany). According to the Endocrine Society clinical practice (ESC) guidelines (22) and the UK Scientific Advisory Committee on Nutrition (SACN) (23), VD status was defined as follows: severely deficient for 25(OH)D <25 nmol/L, deficient for 25 nmol/L≤ 25(OH)D <50 nmol/L, insufficient for 50 nmol/L ≤25(OH)D <75 nmol/L, and sufficient for 25(OH)D ≥75 nmol/L. The Institute of Medicine (IOM) established serum VD values ≥50 nmol/L as sufficient, values between 30 and 50 nmol/L as insufficiency, and values <30 nmol/L as deficiency (24). eGFR was estimated using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (25). In addition, CKD stage was evaluated according to the K/DOQI guidelines.

Kidney Histopathology

Routine examination of every renal biopsy specimen was performed by light microscopy, electron microscopy, and immunofluorescence. Glomerular, tubulointerstitial, and vascular lesions were scored according to the DKD pathologic classification (26). The glomerular classifications were as follows: class I, glomerular basement membrane (GBM) thickening; class IIa, mild mesangial expansion; class IIb, severe mesangial expansion; class III, nodular sclerosis; and class IV, global glomerulosclerosis in >50% of glomeruli. Semi-quantitative scores for interstitial fibrosis and tubular atrophy (IFTA) were obtained according to the affected proportion of the tubulointerstitial compartment (0, none; 1, <25%; 2, 25%–50%; and 3, >50%), and the scale of interstitial inflammation (0, absent; 1, infiltration only in areas related to IFTA; and 2, infiltration in areas without IFTA). Scores for vascular lesions were based on large-vessel arteriosclerosis and arteriolar hyalinosis. Semi-quantitative rank for the intensity of IgG, IgA, IgM, complement 1q (C1q), C3, and C4 staining in each renal tissue section by direct immunofluorescence was classified into four categories (0, negative; 1, weak staining; 2, moderate staining; and 3, strong staining). Any scoring differences between two pathologists were repeatedly reviewed until a consensus was obtained.

Kidney Outcomes

The primary outcome was the composite kidney outcome, defined as a double increase in serum creatinine (D-Scr) from baseline values or the occurrence of ESKD. ESKD was defined as the initiation of maintenance dialysis or kidney transplantation. Patients who did not reach the endpoint were recorded using the information of their last follow-up visit. Survival time was calculated from the enrollment to the occurrence of the event

or the last follow-up. Patient visits usually occurred at intervals of 3–6 months except for those with CKD stage 3 and stage 4 who were under close observation and followed at 1–3 months.

Statistical Analysis

We divided the study population into tertiles according to baseline serum 25(OH)D level. Data were presented as mean ± SD, median and interquartile range, or percentage. As appropriate, comparisons between groups were performed using one-way analysis of variance (ANOVA), Kruskal–Wallis test, or $\chi 2$ test. Pearson's or Spearman correlations were calculated to characterize the associations between baseline characteristics and serum 25(OH)D level. Kaplan–Meier analysis and the log-rank test were used to assess renal survival differences among groups. Hazard ratios for the serum 25(OH)D level with CKD progression were estimated using Cox proportional hazards regression models with follow-up time. The assumption of proportionality was tested using Schoenfeld residuals and interaction terms with time for each exposure variable and covariate. In addition, multiple covariables were adjusted. In addition, we further performed sensitivity analyses with TWA of the serum 25(OH)D level in 75 participants to reinforce the findings. Kaplan–Meier analysis and Cox proportional hazards regression models were performed to evaluate the effect of TWA of the serum 25 (OH)D level on renal outcomes. A p-value of <0.05 was considered statistically significant. All statistics were done in IBM SPSS v.24.0 and R v.4.0.2.

RESULTS

Baseline Clinical and Pathologic Characteristics According to Tertiles of the Serum 25(OH)D Level

In total, 182 patients with T2DM with DKD (141, 78%) and NDKD (41, 22%) were enrolled in this study (**Figure 1**). During a median follow-up time of 42 months (IQR, 24; 62 months), 70 incident kidney outcomes were identified. The clinical characteristics of the cases divided into two groups according to the later development of renal endpoints were summarized in **Table 1**. The mean age was 52 ± 11 years old, and most were male (75%). CKD stages and pathological types were significantly distributed between the two groups (no incidence versus the incidence of renal endpoints, $P < 0.001$). Compared with patients without renal outcomes, significantly higher levels of systolic blood pressure, urinary protein, serum creatinine (Scr), blood urea nitrogen (BUN), total cholesterol, LDL-C, HDL-C, C3, C4, PTH, serum NAGL, urinary NAGL, and 24-h uNAG were observed in patients with D-Scr or development of ESKD, along with lower levels of eGFR, serum albumin, hemoglobin, 25(OH)D, and serum calcium. Moreover, RAASi and oral hypoglycemic agents were significantly more prevalent among patients without D-Scr or ESKD. Insulin, β-blocker, and CCB were used more in patients with D-Scr or ESKD.

The clinical characteristics of the study population according to tertiles of the serum 25(OH)D levels are presented in **Table 2**. The median serum 25(OH)D level was 26 (IQR, 14; 39) nmol/L in the study participants. During follow-up, the incidence of composite kidney outcomes in the lowest tertile was the highest among groups ($P < 0.001$), with 35 patients (58%) progressing to ESKD and four patients (6.7%) progressing to D-Scr from the time of renal biopsy. Consistently, kidney function was better when the serum 25(OH)D level was higher, wherein eGFR was significantly increased in the highest tierce, which had the lowest levels of Scr, BUN, and uric acid (all $P < 0.05$). In addition, blood pressure (including systolic blood pressure, diastolic blood pressure, and mean arterial pressure), CKD stages, 24-h urinary protein, levels of TC, LDL-C, and urinary NAGL reduced, whereas levels of serum albumin, hemoglobin, serum calcium, and 24-h urinary calcium rose across the increasing tertile of serum 25(OH)D (all $P < 0.05$). Moreover, significant differences in the current use of RAASi, oral hypoglycemic agents, diuretic, and statins were observed among the tertiles at baseline (all $P < 0.05$).

In terms of pathological types, patients with DKD had significantly lower serum 25(OH)D levels than those with NDKD ($P < 0.01$, Wilcoxon test, **Figure 2A**). Moreover, there was a significant decrease in 25(OH)D at stage G1 relative to later CKD stages (G1 vs. G3a: $P < 0.05$; G1 vs. G3b: $P < 0.01$; G1 vs. G4: $P < 0.05$). Moreover, only eight (4.4%) patients and 15 (8.2%) patients showed sufficient (≥75 nmol/L) and insufficient 25(OH) D levels (50–75 nmol/L) according to the ESC guideline, with 12.6% exhibiting 25(OH)D sufficiency (≥50 nmol/L) according to the IOM guideline. The prevalence of VD deficiency (25–50 nmol/L) in patients with DKD was higher than that in patients with NDKD (51.5% vs. 31.7%), whereas VD severe deficiency (<25 nmol/L) ratios were similar between two groups (40% vs. 41.5%) in accordance with the ESC and SACN guidelines (**Figure 2B**). In addition, with the standard of IOM guideline, 64.5% of patients with DKD were at risk of deficiency relative to bone health (<30 nmol/L), higher than patients with NDKD (39%) (**Figure 2C**). **Table 3** displays the baseline pathological features of the recruited study population, both overall and stratified by the serum 25(OH)D level. DKD was common in the lowest tertile, whereas NDKD in the highest. In addition, there were significant differences in the glomerular class of DKD, pathological classification of NDKD, and vascular lesion score among groups (all $P < 0.05$). Moreover, patients in the lowest tertile of serum 25(OH)D tended to have a greater proportion of glomerular IgM, C3, and C4 deposition, especially in DKD cases (all $P < 0.05$). On the other hand, those with the highest tertile of serum 25(OH)D tended to have stronger staining of glomerular IgA deposition, especially in NDKD cases (all $P < 0.05$).

Correlation Between the Serum 25(OH)D Level and Clinicopathological Characteristics

On further analyses by Spearman test (**Table 4**), the serum 25 (OH)D level was negatively correlated with proteinuria ($r = -0.62$, $P < 0.001$), Scr ($r = -0.26$, $P < 0.001$), BUN ($r = -0.17$, $P = 0.02$), TC ($r = -0.48$, $P < 0.001$), LDL-C ($r = -0.46$, $P < 0.001$), HDL-C

TABLE 1 | Baseline characteristics and laboratory data for all enrolled patients.

Parameter	Total (n = 182)	D-Scr or ESKD (n = 70)	No D-Scr and no ESKD (n = 112)	*P*-value
Age (years)	52 ± 11	49 ± 11	54 ± 11	**0.005**
Gender (male/female)	137/45	55/15	82/30	0.52
Duration of diabetes (years)	8.0 (3.0, 14.0)	9.0 (3.3, 14.5)	8.0 (2.0, 14.5)	0.73
Composite renal outcome (%)	70 (38)	70 (100)	0 (0)	**<0.001**
Double of serum creatinine (%)	7 (3.8)	7 (10)	0 (0)	**<0.001**
ESKD (%)	63 (35)	63 (90)	0 (0)	**<0.001**
Comorbid disease				
CKD stage (1/2/3a/3b/4)	43/60/23/27/29	8/19/10/12/21	35/41/13/15/8	**<0.001**
DKD/NDKD (cases)	141/41	68/2	73/39	**<0.001**
Diabetic retinopathy (%)	45 (25)	21 (30)	24 (21)	0.26
Diabetic neuropathy (%)	13 (7.1)	7 (10.0)	6 (5.0)	0.38
Cardiovascular diseases (%)	17 (9.3)	2 (2.8)	15 (13.4)	**0.03**
Hypertension (%)	136 (75)	51 (73)	85 (76)	0.78
Clinical parameter				
Body mass index (kg/m2)	25 (23, 27)	25 (23, 27)	25 (23, 27)	0.42
SBP (mmHg)	140 (130, 157)	144 (133, 163)	139 (126, 154)	**0.050**
DBP (mmHg)	85 (74, 94)	85 (78, 95)	83 (74, 92)	0.20
Laboratory parameter				
Urinary protein (g/d)	2.9 (1.3, 7.0)	5.5 (2.9, 9.0)	2.0 (0.8, 5.8)	**<0.001**
eGFR (ml/min/1.73 m^2)	65 (38, 88)	51 (28, 73)	72 (47, 97)	**<0.001**
BUN (mmol/L)	8.9 (6.5, 11.6)	6.6 (5.0, 9.0)	9.9 (7.5, 11.6)	**<0.001**
Scr (μmol/L)	108 (82, 171)	145 (100, 213)	97 (71, 133)	**<0.001**
Uric acid (mmol/L)	369 (316, 425)	388 (335, 442)	357 (315, 418)	0.08
Serum albumin (g/L)	32 ± 7.9	30 ± 7.2	33 ± 8.1	**0.01**
ALP (U/L)	83 (61, 105)	88 (70, 105)	78 (59, 102)	0.28
Fasting blood glucose (mmol/L)	6.4 (4.8, 8.3)	6.9 (5.1, 8.3)	6.3 (4.6, 8.2)	0.32
Glycosylated hemoglobin (%)	7.1 (6.3, 8.1)	6.7 (6.2, 7.7)	7.2 (6.5, 8.6)	**0.04**
Triglyceride (mmol/L)	1.5 (1.1, 2.3)	1.7 (1.1, 2.3)	1.5 (1.1, 2.5)	0.70
Total cholesterol (mmol/L)	5.0 (4.0, 6.0)	5.3 (4.4, 6.4)	4.8 (3.8, 5.9)	**0.02**
LDL-C (mmol/L)	3.2 (2.6, 3.9)	3.5 (2.8, 4.3)	3.1 (2.5, 3.7)	**0.008**
HDL-C (mmol/L)	1.04 (0.87, 1.31)	1.10 (0.93, 1.43)	1.00 (0.85, 1.24)	**0.04**
Hemoglobin (g/L)	115 ± 23	106 ± 18	120 ± 24	**< 0.001**
IgA (g/L)	2.4 (1.8, 3.0)	2.4 (2.0, 3.0)	2.4 (1.7, 3.0)	0.44
IgG (g/L)	9.6 (7.6, 12)	9.5 (7.1, 11)	9.9 (7.7, 12)	0.28
C3 (g/L)	1.05 (0.95, 1.18)	1.06 (0.98, 1.21)	1.03 (0.90, 1.17)	**0.04**
C4 (g/L)	0.30 ± 0.10	0.31 ± 0.08	0.27 ± 0.09	**0.02**
PTH (pg/ml)	44 (27, 70)	49 (32, 82)	37 (24, 53)	**0.01**
25(OH)D (nmol/L)	26 (14, 39)	16 (10, 30)	32 (20, 46)	**<0.001**
Serum calcium (mmol/L)	2.13 (2.00, 2.23)	2.07 (1.96, 2.17)	2.16 (2.04, 2.27)	**0.004**
Serum phosphorus (mmol/L)	1.23 (1.12, 1.38)	1.26 (1.15, 1.42)	1.22 (1.12, 1.37)	0.37
24-h urinary calcium (mmol/d)	1.60 (0.88, 3.18)	1.47 (0.72, 2.66)	1.62 (0.94, 3.64)	0.24
24-h urinary phosphorus (mmol/d)	16.7 (12.3, 23.0)	16.5 (13.0, 19.6)	16.7 (11.6, 23.6)	0.86
Serum NAGL (ng/mL)	218 (141, 304)	243 (183, 414)	180 (126, 266)	**0.003**
Urinary NAGL (ng/mL)	53 (25, 92)	78 (25, 194)	25 (25, 71)	**<0.001**
24-h uNAG (U/L)	15 (10, 24)	20 (14, 28)	14 (9.3, 23)	**0.002**
RBP (mg/L)	60 (43, 73)	66 (55, 84)	56 (40, 69)	**0.002**
Medications				
RAAS inhibitor (%)	147 (81)	46 (66)	101 (90)	**< 0.001**
Oral hypoglycemic agents (%)	90 (50)	24 (34)	66 (59)	**0.002**
Insulin (%)	129 (71)	59 (84)	70 (63)	**0.003**
β-blocker (%)	52 (29)	29 (41)	23 (21)	**0.004**
Diuretic (%)	21 (12)	8 (11)	13 (12)	1.00
CCB (%)	118 (65)	54 (77)	64 (57)	**0.01**
Statins (%)	78 (43)	27 (39)	51 (46)	0.44
Calcium supplements (%)	30 (16)	15 (21)	15 (13)	0.22

DKD, diabetic kidney disease; NDKD, non-diabetic kidney disease; D-Scr, doubling of serum creatinine level; ESKD, end-stage kidney disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; Scr, serum creatinine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol; IgA, immunoglobulin A; IgG, immunoglobulin G; C3, complement 3; C4, complement 4; 24-h UV, 24-h urinary volume; PTH, parathyroid hormone; 25(OH)D, 25-hydroxy vitamin D; RAAS, renin-angiotensin-aldosterone system; CCB, calcium-channel blocker.

Data were presented as the mean ± standard, the median with interquartile range, or counts and percentages. A two-tailed P < 0.05 was considered statistically significant and presented in bold.

(r= −0.19, $P = 0.01$), PTH (r = −0.20, $P = 0.02$), serum phosphorus (r = −0.24, $P = 0.008$), serum C4 level (r = −0.17, $P = 0.03$), 24-h uNAG (r = −0.49, $P < 0.001$), CKD stage (r = −0.26, $P < 0.001$), and vascular lesion score (r = −0.16, $P = 0.04$), whereas it was positively correlated with eGFR (r = 0.26, $P < 0.001$), serum albumin (r = 0.66, $P < 0.001$), serum calcium (r = 0.28, $P = 0.002$), serum IgG (r = 0.46, $P < 0.001$), 24-h urinary calcium (r = 0.28, $P = 0.002$), and 24-h urinary phosphorus (r = 0.24, $P = 0.008$).

TABLE 2 | Clinical characteristics and laboratory findings of all enrolled patients according to tertiles of vitamin D level.

Parameter	Tertiles of the serum 25(OH)D level (nmol/L)			*P*-value
	T1 (≤17) (n = 60)	T2 (17-35) (n = 62)	T3 (>35) (n = 60)	
Age (years)	51 ± 12	52 ± 12	54 ± 9.7	0.32
Gender (male/female)	45/15	45/17	47/13	0.79
Comorbid disease				
CKD stage (1/2/3a/3b/4)	7/17/11/12/13	15/20/7/10/10	21/23/5/5/6	**0.001**
Duration of diabetes (years)	8.5 (3.0, 14)	10 (4.3, 12)	7.0 (3.8, 15)	0.91
Diabetic retinopathy (%)	19 (32)	15 (24)	11 (18)	0.25
Diabetic neuropathy (%)	7 (12)	3 (4.8)	3 (5.0)	0.29
Hypertension (%)	39 (65)	49 (79)	48 (80)	0.11
Cardiovascular diseases (%)	1 (1.7)	8 (13)	8 (13)	**0.04**
Clinical parameter				
BMI (kg/m^2)	25 (23, 27)	24 (23, 27)	26 (24, 28)	0.14
SBP (mmHg)	146 (136, 167)	140 (127, 155)	135 (127, 145)	**0.005**
DBP (mmHg)	87 (79, 99)	83 (72, 90)	81 (73, 93)	**0.01**
MAP (mmHg)	109 ± 16	102 ± 14	101 ± 12	**0.002**
Laboratory parameter				
Urinary protein excretion (g/d)	6.9 (4.7, 11)	2.9 (1.7, 5.6)	1.1 (0.31, 2.7)	**<0.001**
eGFR (ml/min/1.73 m^2)	53 (32, 72)	66 (38, 87)	78 (55, 99)	**0.001**
BUN (mmol/L)	9.3 (7.4, 12)	9.5 (6.5, 13)	7.7 (5.7, 11)	**0.04**
Scr (μmol/L)	129 (101, 198)	108 (82, 171)	93 (71, 124)	**0.001**
Uric acid (mmol/L)	350 (314, 415)	397 (347, 472)	351 (312, 417)	**0.02**
Serum albumin (g/L)	26 ± 6.6	33 ± 6.1	38 ± 5.9	**<0.001**
ALP (U/L)	88 (67, 106)	74 (61, 92)	87 (62, 100)	0.44
Serum 25(OH)D (nmol/L)	10 (7.8, 14)	26 (22, 30)	47 (39, 60)	**<0.001**
Serum calcium (mmol/L)	1.97 (1.89, 2.11)	2.15 (2.04, 2.21)	2.24 (2.15, 2.30)	**<0.001**
Serum phosphorus (mmol/L)	1.22 (1.06, 1.38)	1.27 (1.15, 1.46)	1.19 (1.12, 1.34)	0.25
24-h urinary calcium (mmol/d)	0.99 (0.66, 2.24)	1.45 (0.86, 2.28)	2.41 (1.40, 4.36)	**0.002**
24-h urinary phosphorus (mmol/d)	12.3 (9.0, 16.9)	17.9 (14.2, 23.9)	17.1 (13.2, 24.0)	**0.001**
FBG (mmol/L)	6.6 (5.1, 10)	6.2 (4.6, 8.2)	6.3 (4.7, 7.9)	0.44
HbA1c (%)	6.8 (6.1, 8.1)	7.1 (6.5, 8.4)	7.1 (6.3, 8.0)	0.49
TG (mmol/L)	1.7 (1.2, 2.6)	1.6 (1.3, 2.4)	1.4 (1.0, 2.1)	0.12
TC (mmol/L)	5.7 (5.0, 7.1)	5.2 (4.2, 6.0)	4.1 (3.5, 4.9)	**<0.001**
LDL-C (mmol/L)	3.8 (3.1, 4.9)	3.3 (2.7, 4.0)	2.6 (2.0, 3.2)	**<0.001**
HDL-C (mmol/L)	1.11 (0.97, 1.48)	0.99 (0.84, 1.24)	1.01 (0.86, 1.22)	**0.02**
Hemoglobin (g/L)	105 ± 20	115 ± 23	123 ± 21	**<0.001**
PTH (pg/mL)	57 (34, 100)	42 (28, 62)	36 (23, 46)	**0.007**
Serum IgA (g/L)	2.5 (1.9, 3.1)	2.2 (1.7, 2.7)	2.5 (1.8, 3.1)	0.16
Serum IgG (g/L)	7.5 (6.1, 9.9)	10 (7.8, 13)	11 (9.1, 14)	**<0.001**
Serum C3 (g/L)	1.08 (0.98, 1.20)	1.03 (0.86, 1.13)	1.04 (0.94, 1.19)	0.10
Serum C4 (g/L)	0.31 ± 0.09	0.26 ± 0.09	0.28 ± 0.08	**0.01**
Serum NAGL (ng/mL)	228 (115, 390)	222 (171, 362)	159 (135, 250)	0.21
Urinary NAGL (ng/mL)	95 (57, 190)	53 (25, 77)	25 (25, 38)	**<0.001**
24-h uNAG (U/L)	22 (18, 36)	15 (10, 25)	12 (8.2, 17)	**<0.001**
RBP (mg/L)	58 (44, 71)	62 (48, 76)	58 (41, 72)	0.57
Medications				
RAAS inhibitor (%)	42 (70)	53 (86)	52 (87)	**0.04**
Oral hypoglycemic agents (%)	17 (28)	34 (55)	39 (65)	**<0.001**
Insulin (%)	47 (78)	45 (73)	37 (62)	0.12
β-blocker (%)	22 (37)	17 (27)	13 (22)	0.19
Diuretic (%)	13 (22)	5 (8.1)	3 (5.0)	**0.01**
CCB (%)	44 (73)	38 (61)	36 (60)	0.24
Statins (%)	30 (50)	30 (48)	18 (30)	**0.048**
Calcium supplements (%)	13 (22)	12 (19)	5 (8.3)	0.11

(Continued)

TABLE 2 | Continued

Parameter	Tertiles of the serum 25(OH)D level (nmol/L)			*P*-value
	T1 (≤17) (n = 60)	T2 (17-35) (n = 62)	T3 (>35) (n = 60)	
Progression				
Composite renal outcome (%)	39 (65)	20 (32)	11 (18)	**<0.001**
D-Scr (%)	4 (6.7)	1 (1.6)	2 (3.3)	0.31
ESKD (%)	35 (58)	19 (31)	9 (15)	**<0.001**

CKD, chronic kidney disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP = (systolic blood pressure+2×diastolic blood pressure)/3; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; Scr, serum creatinine; ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; PTH, parathyroid hormone; IgA, immunoglobulin A; IgG, immunoglobulin G; C3, complement 3; C4, complement 4; NAGL, neutrophil gelatinase-associated lipocalin; uNAG, urinary N-acetyl-β-D glucosaminidase; RBP, retinol-binding protein; RAAS, renin-angiotensin-aldosterone system; CCB, calcium-channel blocker. D-Scr, doubling of serum creatinine level; ESKD, end-stage kidney disease. Data were presented as the mean ± standard, the median with interquartile range, or counts and percentages. A two-tailed P < 0.05 was considered statistically significant and presented in bold.

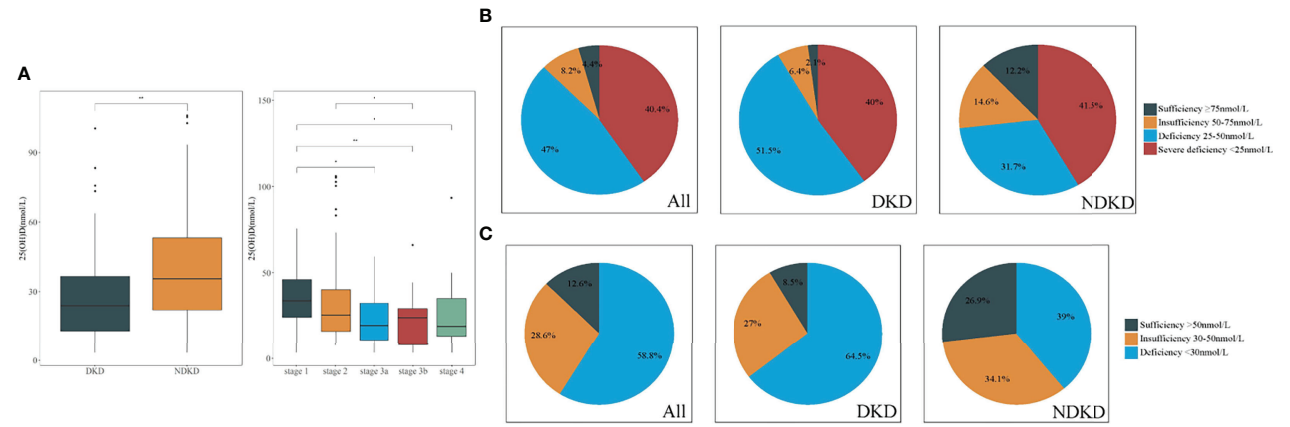

FIGURE 2 | Comparison of vitamin D levels in patients with DKD and NDKD. **(A)** Significant difference was obtained in vitamin D levels between DKD and NDKD, in different stages of CKD, respectively. **(B, C)**. Vitamin D status in all patients with DKD and NDKD, respectively. **(B)** Four categories according to the Endocrine Society clinical practice (ESC) guidelines and the UK Scientific Advisory Committee on Nutrition (SACN). **(C)** Three categories according to the Institute of Medicine (IOM). *$P < 0.05$ between all groups. **$P < 0.01$ between groups. DKD, diabetic kidney disease; NDKD, non-diabetic kidney disease; 25(OH)D, 25-hydroxyvitamin D.

Effect of the Serum 25(OH)D Level on Kidney Outcomes

In the Kaplan–Meier survival analysis (**Figure 3**), the cumulative incidence of kidney outcomes decreased across increasing tertiles of serum 25(OH)D ($P < 0.001$), which suggested that patients with a lower serum 25(OH)D level had a worse kidney outcome. More specifically, in pairwise comparison using the Log-rank test, the *P*-value was 0.001 (the lowest vs. middle tertile), <0.001 (the lowest vs. highest tertile), and 0.008 (the middle vs. highest tertile), respectively. The association between the 25(OH)D level and risks for composite kidney outcomes were further determined by Cox proportional hazards regression model (**Table 5**). In unadjusted models, compared with the highest tertile, the risk of kidney outcomes was higher in the lowest tertile [HR, 6.3 (3.2, 12.4), $P < 0.001$] and also relatively higher in the middle tertile [HR, 2.6 (1.2, 5.4), $P = 0.01$] (model 1 in **Table 5**). After adjustment for baseline eGFR, we also observed a significantly greater risk for CKD progression in the lowest tertile of the serum 25(OH)D level [HR, 5.2 (2.5, 10.7), $P < 0.001$] compared with the highest tertile of the serum 25(OH)D level (model 2 in **Table 5**). This increased risk remained in the lowest tierce, even after extensive adjustment for baseline age, gender, HbA1c, 24-h urinary protein, systolic blood pressure, use of RAASi, oral hypoglycemic agents, and insulin [HR, 3.2 (1.3, 7.8), $P = 0.01$, model 3 in **Table 5**, **Figure 4**].

Effect of TWA of the Serum 25(OH)D Level on Kidney Outcomes

Seventy-five patients received serum 25(OH)D measurements from two to nine times during follow-up. To substantiate our findings, we calculated the TWA of serum 25(OH)D in these 75 patients and further performed sensitivity analyses. We divided the patients into tertiles according to TWA of the serum 25(OH) D level (**Supplementary Tables 1, 2**). During a median follow-up time of 41 months (IQR, 24; 52 months), 31 outcome events occurred, including four events of D-Scr and 27 events of ESKD. Compared with the lowest tertile of TWA, Kaplan–Meier analyses demonstrated that the cumulative incidence of kidney outcomes was significantly lower in the highest tertile of TWA of the serum 25(OH)D level ($P < 0.001$) (**Figure 5**). In addition, we

TABLE 3 | Pathological features of all enrolled patients according to tertiles of vitamin D level.

Pathological feature	Total (n = 182)	Tertiles of the serum 25(OH)D level (nmol/L)			*P*-value
		T1 (≤17) (n = 60)	T2 (17-35) (n = 62)	T3 (>35) (n = 60)	
DKD/NDKD (cases)	141/41	53/7	49/13	39/21	**0.009**
Glomerular class of DKD (I/IIa/IIb/III/IV)	0/12/33/73/23	0/1/10/35/7	0/4/9/25/11	0/7/14/13/5	**0.01**
Pathological classification of NDKD					
IgAN/MCD/FSGS/MN/LN/CGN (cases)	18/1/9/9/1/3	1/0/2/4/0/0	3/0/3/4/1/2	14/1/4/1/0/1	**0.004**
IFTA Score (0/1/2/3)	12/65/37/66	2/18/12/26	6/21/14/21	4/26/12/19	0.18
DKD subtype	6/40/34/61	2/14/12/25	2/13/14/20	2/13/8/16	0.74
NDKD subtype	6/25/3/5	0/4/0/1	4/8/0/1	2/13/3/3	0.14
Interstitial inflammation (0/1/2/3)	23/101/55/1	10/25/22/1	5/43/14/0	8/33/19/0	0.63
DKD subtype	17/79/44/1	8/24/20/1	4/33/12/0	5/22/12/0	0.65
NDKD subtype	6/22/11/0	2/1/2/0	1/10/2/0	3/11/7/0	0.80
Vascular lesion Score (0/1/2)	38/58/84	8/21/29	11/16/35	19/21/20	**0.02**
DKD subtype	13/44/84	5/19/29	2/12/35	6/13/20	0.08
NDKD subtype	25/14/0	3/2/0	9/4/0	13/8/0	0.89
Global sclerosis, %	25 (7.3, 42)	26 (13, 39)	22 (6.9, 50)	28 (11, 46)	0.74
DKD subtype	25 (9.6, 45)	25 (12, 39)	20 (6.9, 46)	30 (12, 46)	0.62
NDKD subtype	21 (0.0, 33)	32 (31, 43)	15 (6.7, 33)	20 (0.0, 33)	0.44
Glomerular IgG deposition (0/1/2/3)	119/45/9/6	34/17/6/1	40/16/2/3	45/12/1/2	0.16
DKD subtype	93/40/7/1	32/16/4/1	33/14/2/0	28/10/1/0	0.41
NDKD subtype	26/5/2/5	2/1/2/0	7/2/0/2	17/2/0/2	0.18
Glomerular IgM deposition (0/1/2/3)	39/31/42/67	6/8/13/31	17/12/9/23	16/11/20/13	**0.002**
DKD subtype	29/22/33/57	5/5/13/30	14/11/6/18	10/6/14/9	**0.002**
NDKD subtype	10/9/9/10	1/3/0/1	3/1/3/5	6/5/6/4	0.47
Glomerular IgA deposition (0/1/2/3)	103/32/19/25	34/14/6/4	41/5/10/5	28/13/3/16	**0.046**
DKD subtype	88/31/14/8	31/13/6/3	35/5/6/3	22/13/2/2	0.51
NDKD subtype	15/1/5/17	3/1/0/1	6/0/4/2	6/0/1/14	**0.046**
Glomerular C3 deposition (0/1/2/3)	93/17/16/53	19/8/6/25	38/5/5/13	36/4/5/15	**0.002**
DKD subtype	73/13/14/41	15/7/6/25	34/3/5/7	24/3/3/9	**<0.001**
NDKD subtype	20/4/2/12	4/1/0/0	4/2/0/6	12/1/2/6	0.21
Glomerular C4 deposition (0/1/2/3)	136/18/13/12	39/6/6/7	45/10/3/3	52/2/4/2	**0.04**
DKD subtype	103/16/11/11	34/6/6/7	35/9/3/2	34/1/2/2	**0.049**
NDKD subtype	33/2/2/1/	5/0/0/0	10/1/0/1	18/1/2/0	0.64
Glomerular C1q deposition (0/1/2/3)	112/27/21/19	31/9/9/9	38/13/3/7	43/5/9/3	0.11
DKD subtype	87/19/18/17	27/8/9/9	34/7/2/6	26/4/7/2	0.10
NDKD subtype	25/8/3/2	4/1/0/0	4/6/1/1	17/1/2/1	**0.04**

DKD, diabetic kidney disease; NDKD, non-diabetic kidney disease; IgAN, IgA nephropathy; MCD, minimal change disease; FSGS, focal segmental glomerulosclerosis; MN, membranous nephropathy; LN, lupus nephritis; CGN, crescentic glomerulonephritis; IFTA, interstitial inflammation.
A two-tailed P < 0.05 was considered statistically significant and presented in bold.

created a Cox proportional hazards regression model with tertiles of TWA of the serum 25(OH)D level in the same manner as above. Patients in the lowest tertile of TWA of the serum 25(OH)D level were associated with a higher risk for CKD progression [HR, 9.5 (2.8, 32.7), $P < 0.001$, model 1 in **Table 6**] compared with those in the highest tertile. This association remained significant after adjustment for baseline eGFR in model 2 [HR, 8.6 (2.5, 30.1), $P = 0.001$ for the lowest tertile, model 2 in **Table 6**]. After adjustment for baseline age, gender, HbA1c, 24-h urinary protein, systolic blood pressure, use of RAASi, oral hypoglycemic agents, and insulin, the risk for kidney outcomes increased with the reduction of 25(OH)D level (P for trend = 0.02, model 3 in **Table 6**).

DISCUSSION

The current study was specifically powered on the serum 25(OH) D level and T2DM with CKD. The principal finding of this study is that lower serum 25(OH)D level was significantly associated with an increased risk of CKD progression in patients with T2DM. This association was independent of other established important covariables, including baseline eGFR, age, HbA1c, 24-h urinary protein, blood pressure, use of RAASi, oral hypoglycemic agents, and insulin. In addition, these relationships remained robust with further sensitivity analysis of data with TWA of the serum 25(OH) D level, showing an independent association between lower TWA of the serum 25(OH)D level and an unfavorable kidney outcome in patients with T2DM with CKD.

Surveys conducted in Chinese cities among patients with T2DM reported the proportions of VD deficiency were about 62.7%–83.5% (27–29). In addition, VD deficiency may be a prominent element of CKD due to that reduced CYP27B1 activity in human renal PTECs inhibits the production of 1,25 (OH)2D and impairs the function of reabsorption of 25(OH)D (6, 30). Here, we reported 87.4% [25(OH)D <50 nmol/L], 40.4% [25 (OH)D <25 nmol/L], or 58.8% [25(OH)D <30 nmol/L] of patients with T2DM with CKD in Nanjing, which is located in eastern

TABLE 4 | Correlations between serum 25(OH)D and clinicopathological parameters.

Parameter	25(OH)D	
	r	*P*-value
Clinical parameter		
Urinary protein excretion (g/d)	−0.62	**<0.001**
eGFR (ml/min/1.73 m²)	0.26	**<0.001**
BUN (mmol/L)	−0.17	**0.02**
Scr (μmol/L)	−0.26	**<0.001**
Serum albumin (g/L)	0.66	**<0.001**
FBG (mmol/L)	−0.13	0.08
HbA1c (%)	−0.03	0.69
TG (mmol/L)	−0.15	0.05
TC(mmol/L)	−0.48	**<0.001**
LDL-C (mmol/L)	−0.46	**<0.001**
HDL-C (mmol/L)	−0.19	**0.01**
PTH(pg/mL)	−0.20	**0.02**
Serum calcium (mmol/L)	0.28	**0.002**
Serum phosphorus (mmol/L)	−0.24	**0.008**
Serum IgA (g/L)	−0.01	0.85
Serum IgG (g/L)	0.46	**<0.001**
Serum C3 (g/L)	−0.09	0.25
Serum C4 (g/L)	−0.17	**0.03**
Serum NAGL (ng/mL)	−0.08	0.40
Urinary NAGL (ng/mL)	−0.48	**<0.001**
24-h uNAG (U/L)	−0.49	**<0.001**
24-h urinary calcium (mmol/d)	0.28	**0.002**
24-h urinary phosphorus (mmol/d)	0.24	**0.008**
RBP (mg/L)	−0.003	0.97
CKD stage (1/2/3a/3b/4)	−0.26	**<0.001**
Pathological feature		
IFTA Score (0/1/2/3)	−0.12	0.13
Interstitial inflammation (0/1/2/3)	−0.03	0.65
Vascular lesion Score (0/1/2)	−0.16	**0.04**
Global sclerosis, %	0.04	0.63

DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; Scr, serum creatinine; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; IgA, immunoglobulin A; IgG, immunoglobulin G; C3, complement 3; C4, complement 4; NAGL, neutrophil gelatinase-associated lipocalin; uNAG, urinary N-acetyl-β-D glucosaminidase; RBP, retinol-binding protein; IFTA, interstitial inflammation.

A two-tailed P < 0.05 was considered statistically significant and presented in bold.

coastal China (latitude 31°–33°N), had VD deficiency according to those international guidelines. By dividing into pathological subgroups, up to 91.5% of patients with DKD were affected by VD deficiency and insufficiency, presenting decreased the serum 25(OH)D levels than those with NDKD. The findings suggested VD homeostasis might be related to the etiology and pathogenesis of DKD in patients with T2DM. The Third National Health and Nutrition Examination Survey (NHANES III) to assess people with diabetes demonstrated an independent association between VD deficiency and DKD (31). In addition, the insufficiency of VD is more serious when DKD is progressing (32).

Previous studies were mainly focused on T2DM population to explore the relationship of VD levels and the presence of DKD (12, 13, 28). Recent studies have indicated that hypovitaminosis D was associated with a higher risk of developing DKD in T2DM (15). The included population of our current study was patients with T2DM complicated with biopsy-proven CKD, which was allowed to assess the clinical and pathological features in strata of VD levels and better illustrate the relationship between the development of renal function through pre-dialysis stages with different levels of VD. More importantly, our data strongly suggest that in patients with T2DM complicated with CKD, lower serum 25(OH)D levels were associated with an increased risk of CKD progression. The findings are robust because we showed consistent results with baseline and time-updated patterns of 25(OH)D levels. Patients with both lower baseline and TWA of 25(OH)D levels were almost three times risk to CKD progression compared with those with higher 25(OH)D levels after adjustment for multiple risk factors. This is the first study in patients with T2DM with CKD highlighting TWA of 25 (OH)D levels, representing a sensitivity analysis that supports our primary hypothesis regarding the association between lower 25(OH)D levels and adverse kidney-related outcomes. These results might suggest that the long-term maintenance of optimal VD concentrations early in life has been associated with reduced future risk of CKD development in T2DM.

In the present study, we observed that 25(OH)D levels were positively correlated with serum calcium, 24-h urinary calcium and phosphorus excretion, whereas negatively correlated with PTH level and vascular lesion score. The VD endocrine system is

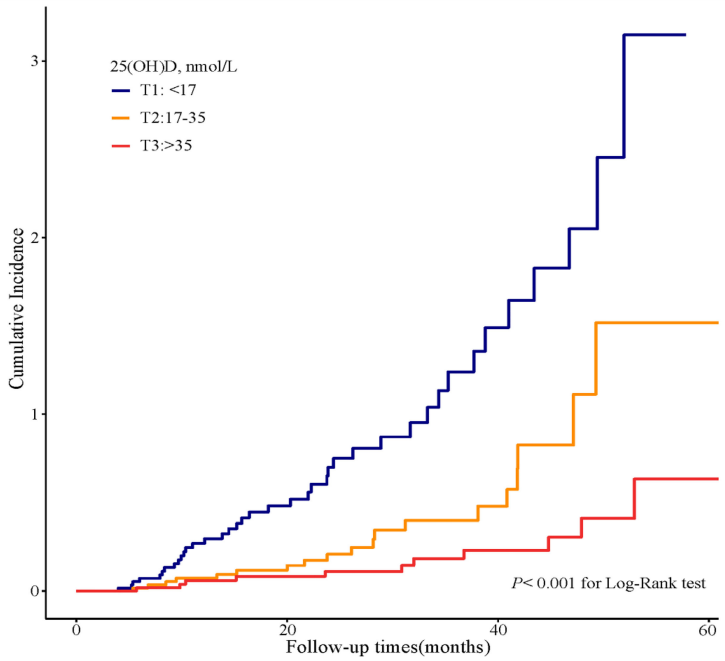

FIGURE 3 | Cumulative incidence of CKD progression in strata of tertiles of the serum 25(OH)D levels. Kaplan–Meier curves comparing different strata of tertiles of the serum 25(OH)D levels in enrolled patients.

critical for human health. VD and its active metabolite are steroid hormones, contributing to regulating the metabolism of calcium and phosphate and playing a critical role in maintaining bone health (33). The best characterized features of CKD associated with VD deficiency are defects in mineral metabolism, including intestinal calcium absorption and renal phosphate excretion (34). Low serum 25(OH)D levels contribute to reduced 1,25(OH)2D levels by providing less substrate for conversion (35). Subsequently, lower 1,25(OH)2D levels reduce calcium absorption from the gastrointestinal tract, promoting PTH secretion, which is associated with abnormal bone remodeling and the propensity to vascular calcifications (30, 34). Therefore, in the present study, the 25(OH)D levels were positively associated with serum calcium and 24-h urinary calcium excretion, whereas negatively correlated with PTH level and vascular lesion score. In addition, VD, PTH, FGF 23, and klotho form a complex endocrine network to maintain phosphate homeostasis (34). Previous studies reported that active VD induces expression of the FGF23 and α-klotho genes to attenuate the pro-aging effects of hyperphosphatemia and maintain the plethora of anti-aging and pro-survival actions of renal and circulating klotho (34). Taken together, in T2D with CKD, low 25(OH)D levels may have a role in electrolyte imbalance and vascular lesions, which need further well-designed studies to elucidate the mutual relationship and their detailed molecular mechanisms.

TABLE 5 | Effect of serum 25(OH)D on renal outcomes.

Variable	Serum 25(OH)D			*P*-value for trend
	T1 (≤17) (n = 60)	T2 (17–35) (n = 62)	T3 (>35) (n = 60)	
Number of events, %	39 (65)	20 (32)	11 (18)	**<0.001**
Model 1 HR (95%CI)	6.3 (3.2, 12.4)	2.6 (1.2, 5.4)	1 [reference]	**<0.001**
***P*-value**	**<0.001**	**0.01**		
Model 2 HR (95%CI)	5.2 (2.5, 10.7)	2.3 (1.1, 4.8)	1 [reference]	**<0.001**
***P*-value**	**<0.001**	**0.04**		
Model 3 HR (95%CI)	3.2 (1.3, 7.8)	1.7 (0.8, 3.9)	1 [reference]	**0.03**
***P*-value**	**0.01**	0.19		

Hazard ratios (HR) and 95% confidence intervals were derived from Cox proportional hazards regression models.
CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; RAAS, renin-angiotensin-aldosterone system; HbA1c, glycosylated hemoglobin; SBP, systolic blood pressure.
Model 1: unadjusted.
Model 2: adjusted for eGFR.
Model 3: Model 2 plus age, gender, HbA1c, 24-h urinary protein, SBP, use of RAAS inhibitor, oral hypoglycemic agents, and insulin.
A two-tailed P < 0.05 was considered statistically significant and presented in bold.

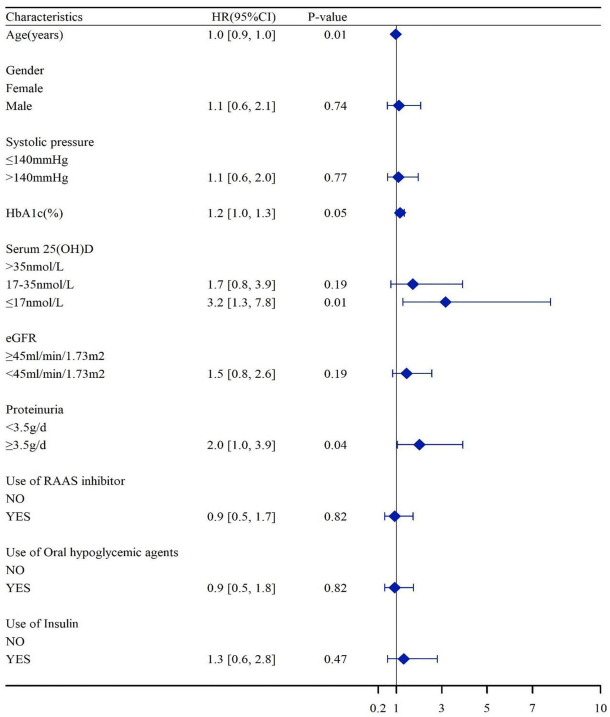

FIGURE 4 | Association of the serum 25(OH)D levels with HR of CKD progressions. Hazard ratios were adjusted for baseline age, gender, HbA1c, 24-h urinary protein, systolic blood pressure, use of RAASi, oral hypoglycemic agents, and insulin. eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin; RAASi, renin-angiotensin-aldosterone system inhibitor.

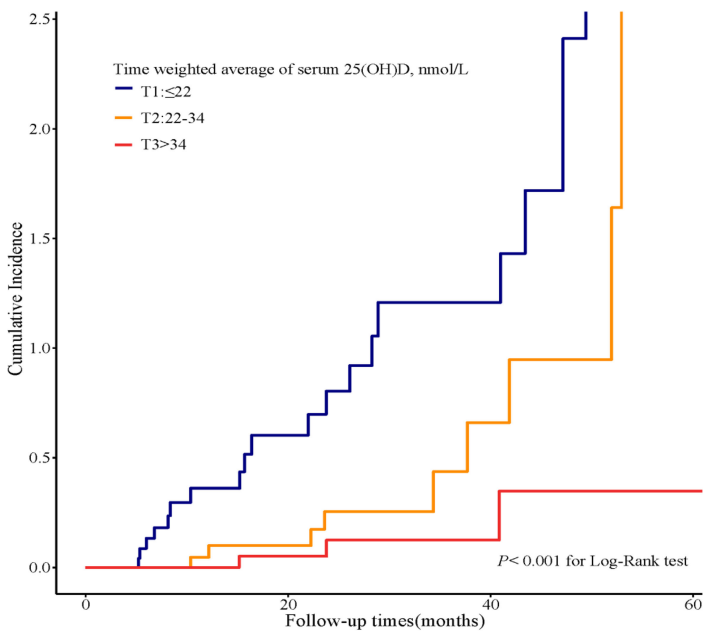

FIGURE 5 | Cumulative incidence of CKD progression in strata of tertiles of TWA of the serum 25(OH)D levels. Kaplan–Meier curves compare different strata of tertiles of TWA of the serum 25(OH)D levels in enrolled patients. TWA, time weighted average.

TABLE 6 | Effect of time weighted average of serum 25(OH)D on renal outcomes.

Variable	TWA of serum 25(OH)D			*P*-value for trend
	T1 (≤22) (n = 25)	T2 (22–34) (n = 25)	T3 (>34) (n = 25)	
Number of events, %	39 (65)	20 (32)	11 (18)	**<0.001**
Model 1 HR (95%CI)	9.5 (2.8, 32.7)	3.2 (0.9, 11.8)	1 [reference]	**<0.001**
***P*-value**	**<0.001**	0.08		
Model 2 HR (95%CI)	8.6 (2.5, 30.1)	2.5 (0.6, 9.7)	1 [reference]	**<0.001**
***P*-value**	**0.001**	0.18		
Model 3 HR (95%CI)	3.7 (0.8, 17.4)	1.0 (0.2, 5.2)	1 [reference]	**0.02**
***P*-value**	0.10	0.96		

Hazard ratios (HR) and 95% confidence intervals were derived from Cox proportional hazards regression models.
TWA, time weighted average; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; RAAS, renin-angiotensin-aldosterone system; HbAc1, glycosylated hemoglobin; SBP, systolic blood pressure.
Model 1: unadjusted.
Model 2: adjusted for eGFR.
Model 3: Model 2 plus age, gender, HbA1c, 24-h urinary protein, SBP, use of RAAS inhibitor, oral hypoglycemic agents, and insulin.
A two-tailed P < 0.05 was considered statistically significant and presented in bold.

Several limitations of our study should be considered. First, the current study is observational in nature. It precludes conclusions concerning causality and cannot exclude the possibility of residual confounding. We conducted an additional multivariable logistic regression analysis with TWA of 25(OH)D levels to further ameliorate the imbalance in potential confounders. However, some bias inherent to retrospective studies may play. Second, this study was conducted in a single center, and the sample size was limited. Third, although serum and 24-h urinary calcium and phosphorus levels, PTH, and renin-angiotensin blocker were analyzed, other potential confounding factors affecting 25(OH) D levels were not included, such as outdoor exercise, sun exposure, nutritional status, seasonal alternation, dietary habits, and bone metabolism markers. In addition, direct measurement of free 25(OH)D and DBP were not routinely performed in clinical practice.

In conclusion, our data suggested that patients with T2DM with a decreased 25(OH)D level had deteriorated renal function. Both lower baseline and TWA of the serum 25(OH)D levels were associated with increased risk of CKD progression in patients with T2DM after adjusting numerous potential confounders, which suggested that the long-term maintenance of optimal VD levels from early in life might be associated with reduced future risk of CKD development in T2DM.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SD designed the research and contributed to the writing. FL analyzed the data and performed statistical analysis. BW, CZ, GN, LS, ZH, and HG reviewed the manuscript. CX and BZ conceived and coordinated the study and had responsibility for its final content. YY is the guarantor of this work who had complete access to all the data in the study and takes ultimate responsibility for the study design and integrity of data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.929598/full#supplementary-material

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Comparisons of the Relationships Between Multiple Lipid Indices and Diabetic Kidney Disease in Patients With Type 2 Diabetes: A Cross-Sectional Study

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Background: Dyslipidemia is a well-recognized risk factor for diabetic kidney disease (DKD) in patients with type 2 diabetes (T2D). Growing evidences have shown that compared with the traditional lipid parameters, some lipid ratios may provide additional information of lipid metabolism. Thus, the present study aimed to investigate which lipid index was most related to DKD.

Methods: This study was a cross-sectional study that enrolled patients with T2D from January 2021 to October 2021. Each participant was screened for DKD, and the diagnostic criterion for DKD is estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 m^2 or urinary albumin-to-creatinine ratio (UACR) ≥ 30 mg/g for 3 months. Fasting blood was collected to determine lipid profiles by an automatic biochemical analyzer, and lipid ratios were calculated based on corresponding lipid parameters. Spearman's correlation analyses were conducted to assess the correlations between lipid indices and kidney injury indices, and binary logistic regression analyses were conducted to explore the relationship between lipid indices and the risk of DKD.

Results: A total of 936 patients with T2D were enrolled in the study, 144 (15.38%) of whom had DKD. The LDL-C/Apo B ratios were positively correlated with eGFR (r = 0.146, $p < 0.05$) and inversely correlated to cystatin C and UACR (r = -0.237 and -0.120, both $p < 0.001$). Multiple logistic regression demonstrated that even after adjusting for other clinical covariates, the LDL-C/Apo B ratios were negatively related to DKD, and the odds ratio (95% confidence interval) was 0.481 (0.275–0.843). Furthermore, subgroup analyses revealed that compared with patients with normal lipid profiles and a high LDL-C/Apo B ratio, the odds ratio of DKD in patients with normal lipid metabolism and a low LDL-C/Apo B ratio was 2.205 (1.136-4.280) after adjusting for other clinical covariates.

Conclusion: In patients with T2D, the LDL-c/Apo B ratio was most closely associated with DKD among various lipid indices, and a lower LDL-C/Apo B ratio was associated with increased risks of DKD among patients with T2D.

Keywords: type 2 diabetes, diabetic kidney disease, lipid indices, low-density lipoprotein cholesterol/apolipoprotein B ratio, small dense low-density lipoprotein cholesterol

INTRODUCTION

As one of the major microvascular complications of type 2 diabetes (T2D), diabetic kidney disease (DKD) affects about 20% of patients with T2D (1). The presence of DKD not only is susceptible to progress to end-stage renal disease requiring dialysis (2), but also significantly increases the risk of cardiovascular disease (CVD) (3), thus creating a great impaction on patients with T2D. An integrated approach should be taken for the prevention and control of DKD for the fact that DKD is a multifactorial disease. Among them, lipid metabolism disorder exerts key roles on the pathogenesis of DKD and functions as an important target for DKD prevention and treatment. Dyslipidemia in patients with T2D is typically characterized with high levels of triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C), and low level of high-density lipoprotein cholesterol (HDL-C) (4). Statins, the mainstay of current lipid-lowering drugs, reduce plasma levels of LDL-C and TG through inhibiting cholesterol synthase (5). However, although statins are recommended by all available guidelines and can reduce proteinuria and all-cause mortality, they fail to delay the progression of end-stage renal disease (6). Therefore, it is plausible that it is not fully to reflect the risk of DKD based on the traditional lipid indices, and it is desirable to search for new lipid-lowering therapeutic targets.

In recent years, growing evidences have shown that compared with the traditional lipid indices, apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B) and lipid ratios may provide additional information of lipid metabolism. Apo A1 and Apo B are structural components of HDL-C and atherosclerotic lipoprotein respectively (7), thus the Apo B/Apo A1 ratio is a surrogate maker of the cholesterol balance between atherogenic and antiatherogenic lipoprotein particles. Multiple studies have demonstrated that the Apo B/Apo A1 ratio is closely associated with cardiac vulnerable plaques (8), and in-stent restenosis (9). Zhao et al. also revealed that a high level of Apo B/Apo A1 ratio can predict the progression of DKD (10). Moreover, the TG/HDL-C ratio, HDL-C/Apo A1 ratio and LDL-C/Apo B ratio have drawn extensive attention. TG/HDL-C ratio is identified as an atherosclerosis index, and a longitudinal follow-up study showed that the TG/HDL-C ratio was a predictor of microvascular complications in patients with T2D (11). Other research has shown that the HDL-C/Apo A1 ratio and LDL-C/Apo B ratio are closely related to the deterioration of glycemia and the onset of T2D (12). In a word, these lipid indices may be more conducive to DKD risk stratification in patients with T2D, but few studies have focused on comparing the relationships between these parameters and DKD in patients with T2D. Therefore, the present study was designed to explore which lipid index is optimal-related to DKD in patients with T2D.

METHODS

Study Design and Participants

In this observational cross-sectional study, patients diagnosed with T2D according to the statement of the American Diabetes Association and screened for DKD were enrolled from the inpatient department of the Second Affiliated Hospital of Nantong University from January 2021 to October 2021 (13). The following cases were excluded: type 1 diabetes, secondary diabetes, previous and current malignant tumors, chronic hepatitis and heart failure, acute diabetic complications and other kidney diseases and urinary tract infection. Finally, 936 patients with T2D were included in the present study. After full understanding of the present study protocol, each subject signed written informed consent. The study was in accordance with the Declaration of Helsinki and approved by the medical research ethics committee of the Second Affiliated Hospital of Nantong University.

Basic Data Collection

At enrollment, all subjects completed a questionnaire with the assistance of experienced physicians to collect the demographic data, lifestyle, medication history and diagnosis history of diseases. Body mass index (BMI) was calculated as the weight/height squared. Blood pressure was measured by a standard mercury sphygmomanometer, and the average of three recordings was recorded.

Laboratory Examination and Calculation

After enrollment, fasting blood samples and fresh first-void morning urine samples were taken for measurement of laboratory parameters, urinary albumin and urinary creatinine, respectively. Lipid profiles and urinary creatinine were measured by an automated biochemical analyzer (Model 7600, Hitachi), and lipid ratios were calculated based on corresponding lipid parameters. Urinary albumin level was evaluated by the immunoturbidimetry method (Immage 800, Beckman Coulter). The algorithm of urinary albumin creatinine ratio (UACR) was the ratio of urinary albumin to urinary creatinine. Estimated glomerular filtration rate (eGFR) was calculated based on the CKD-EPI creatinine-cystatin C equation (2012) (14). If a patient with T2D has an eGFR < 60 ml/min/1.73 m^2 or a UACR ≥ 30

mg/g lasted for more than 3 months, a DKD diagnosis can be made (15).

Grouping Criteria

To explore the relationship between low LDL-C/Apo B ratio and the risk of DKD in T2D patients with normal lipid profiles, the total population was divided into A, B, C and D four groups according to lipid profiles and LDL-C/Apo B ratio. In our laboratory, the reference ranges of lipid metabolism parameters TG, TC, HDL-C and LDL-C were respectively 0.2 - 2.0 mmol/L, 2.9 – 6.0 mmol/L, 1.1 – 1.7 mmol/L and 1.55 – 3.35 mmol/L. Therefore, dyslipidemia was defined as any lipid parameter above the normal ranges described above. Then, a low or a high LDL-C/Apo B ratio was defined according to the median of LDL-C/Apo B ratio (2.8804). Ultimately, group A, B, C and D respectively represented normal lipid profile with a high LDL-C/Apo B ratio, normal lipid profile with a low LDL-C/Apo B ratio, abnormal lipid profile with a high LDL-C/Apo B ratio and abnormal lipid profile with a low LDL-C/Apo B ratio.

Statistical Analysis

The clinical characteristics of the participants grouped by DKD status were described by mean ± SD, median (25 and 75% interquartile) and frequencies (percentages) for the normally and skewed distributed continuous variables and the categorical variables, respectively. We adopted Student's t-test to compare differences in normally distributed data, the Mann–Whitney test to compare differences in skewed distributed data and the chi-square test to compare categorical data. Spearman's bivariate correlation analyses were conducted to analyze the correlations of multiple lipid indices with DKD indicators. As the LDL-C/Apo B ratio was the only parameter related to DKD, multivariate logistic regression analyses were performed to investigate the impact of the LDL-C/Apo B ratio on DKD. Furthermore, the differences in the proportion and odds ratio (OR) were compared among group A, B, C and D by the chi-square test and multivariate logistic regression analyses. All analyses were performed using SPSS statistical software 18.0 (IBM SPSS Inc., USA). A value of $p < 0.05$ was defined as statistical significance.

RESULTS

Clinical Characteristics of the Study Participants

Among the recruited 936 T2D patients, patients combined with DKD accounted for 15.38%. As shown in **Table 1**, T2D patients with DKD had older ages, longer diabetic durations, higher systolic blood pressures, a higher prevalence of hypertension, higher prevalence of insulin, and statins users, higher blood urea nitrogen (BUN) levels, creatinine (Cr) levels, uric acid (UA) levels, cystatin C levels, UACR levels, lower eGFRs, and lower LDL-C/Apo B ratios (all $p < 0.05$) than T2D patients without DKD. There were no differences in proportion of males, BMI, diastolic blood pressures, uses of other antidiabetic treatments other than insulin, HbA1c levels and lipid indices other than LDL-C/Apo B ratio between patients with and without DKD ($p > 0.05$).

Relationships Between Lipid Indices and Kidney Damage Indices in Patients With T2D

Table 2 shows that the LDL-C/Apo B ratio was positively correlated with eGFR ($r = 0.146$, $p < 0.05$), and negatively correlated with cystatin C levels and UACR ($r = -0.237$ and -0.120, both $p < 0.05$). Cystatin C levels were significantly correlated with HDL-C levels, Apo B levels, HDL-C/Apo A1 ratios and Apo B/Apo A1 ratios ($r = -0.102$, 0.074, 0.084, -0.129 and 0.116, respectively, all $p < 0.05$). UACR were positively correlated with TG levels and TG/HDL-C ratios ($r = 0.127$ and 0.123, both $p < 0.05$). However, there were no significant correlations between kidney damage indices and total cholesterol (TC) levels and Apo A1 levels (all $p > 0.05$).

Association of the LDL-C/Apo B Ratio With DKD in Patients With T2D

As the LDL-C/Apo B ratio was the only parameter correlated with all kidney damage indices, we thereby constructed multivariate logistic regression analyses to analyze the association between the LDL-C/Apo B ratio and DKD in patients with T2D. As illustrated in **Table 3**, DKD was significantly associated with the LDL-C/Apo B ratio [OR (95% CI), 0.560 (0.409-0.766)] in the basal unadjusted model 0. Even in the fully adjusted model 2, the LDL-C/Apo B ratio was still independently associated with DKD [OR (95% CI), 0.481 (0.275-0.843)].

Proportion and ORs of DKD Based on Subgroup Analyses

There were significant differences in the proportion of patients with DKD among Group A, B, C and D (8.7%, 21.3%, 12.8% and 19.7%, respectively, p for trend < 0.05). In **Table 4**, compared with Group A, the ORs of DKD in Group B, C and D were 2.850 (1.705-4.763), 1.557 (0.855-2.837) and 2.597 (1.538-4.385), respectively. After adjusting for other clinical factors, the ORs of DKD in Group B, C and D were 2.205 (1.136-4.280), 2.315 (1.078-4.974) and 3.513 (1.762-7.004) vs Group A, respectively (**Figure 1**).

DISCUSSION

In the current study, we evaluated the associations between multiple lipid indices with the risk of DKD in patients with T2D. We found that patients with DKD had a lower level of LDL-C/Apo B ratio than patients without DKD. The LDL-C/Apo B ratio was significantly associated with eGFR, UACR and cystatin C. We also demonstrated that the LDL-C/Apo B ratio is independently related to the prevalence of DKD. Moreover, we revealed that even in patients with normal lipid profiles, a low level of LDL-C/Apo B ratio was relevant to an increased risk of DKD.

TABLE 1 | Clinical characteristics of the study participants.

Variables	T2D			*p* value
	Total	Without DKD	With DKD	
n	936	792	144	
Age (years)	57.65 ± 13.60	55.59 ± 13.20	68.98 ± 9.65	<0.001
Male, *n* (%)	533 (56.9)	458 (57.8)	75 (52.1)	0.202
Diabetic duration (years)	6.0 (1.0-10.0)	5.0 (1.0-10.0)	10.0 (5.8-20.0)	<0.001
Smoking history, *n* (%)	85 (9.1)	68 (8.6)	17 (11.8)	0.210
BMI (kg/m^2)	25.62 ± 3.94	25.63 ± 4.04	25.55 ± 3.36	0.842
Hypertension, *n* (%)	345 (36.9)	260 (32.8)	85 (59.0)	<0.001
SBP (mmHg)	134 (123-147)	132 (123-145)	142 (126-157)	<0.001
DBP (mmHg)	81.37 ± 11.04	81.43 ± 10.50	81.08 ± 13.69	0.726
Antidiabetic treatments				
Insulin treatment, *n* (%)	242 (25.9)	182 (23.0)	60 (41.7)	0.001
Metformin, *n* (%)	415 (44.3)	350 (44.2)	65 (45.1)	0.856
Acarbose, *n* (%)	82 (8.8)	68 (8.6)	14 (9.7)	0.632
Insulin-secretagogues, *n* (%)	284 (30.3)	239 (30.2)	45 (31.3)	0.844
Insulin-sensitisers, *n* (%)	77 (8.2)	64 (8.1)	13 (9.0)	0.741
DPP-4 inhibitors, *n* (%)	44 (4.7)	35 (4.4)	9 (6.3)	0.389
SGLT-2 inhibitors, *n* (%)	66 (7.1)	52 (6.6)	14 (9.7)	0.213
Antihypertensive treatments				
CCB, *n* (%)	230 (24.6)	169 (21.4)	61 (42.4)	<0.001
ARB, *n* (%)	188 (20.1)	145 (18.3)	43 (29.9)	0.002
β-blockers, *n* (%)	45 (4.8)	26 (3.3)	19 (13.2)	<0.001
Diuretics, *n* (%)	76 (8.1)	54 (6.8)	22 (15.3)	0.001
Statins medications, *n* (%)	58 (6.2)	35 (4.4)	23 (16.0)	<0.001
HbA1c (%)	9.32 ± 2.16	9.35 ± 2.14	9.14 ± 2.27	0.284
BUN (mmol/L)	5.23 (4.32-6.62)	5.11 (4.20-6.26)	7.13 (5.32-9.16)	<0.001
Cr (umol/L)	56.0 (47.0-67.0)	54.0 (46.0-63.0)	86.0 (68.0-116.0)	<0.001
Serum UA (umol/L)	301.0 (241.0-375.0)	290.5 (232.3-354.8)	380.0 (313.0-472.0)	<0.001
Cystatin C (mg/L)	0.83 (0.67-1.02)	0.78 (0.64-0.93)	1.33 (1.15-1.65)	<0.001
eGFR (ml/min/1.73m^2)	102.93 ± 29.22	110.58 ± 23.22	58.60 ± 19.31	<0.001
UACR (mg/g)	16.25 (8.10-47.65)	13.60 (7.70-31.98)	141.45 (24.15-897.55)	<0.001
TG (mmol/L)	1.64 (1.07-2.67)	1.62 (1.06-2.59)	1.79 (1.14-2.83)	0.357
TC (mmol/L)	4.34 (3.72-5.01)	4.36 (3.74-5.03)	4.27 (3.57-4.96)	0.121
HDL-C (mmol/L)	1.12 (0.95-1.31)	1.12 (0.96-1.31)	1.12 (0.92-1.30)	0.360
LDL-C (mmol/L)	2.73 ± 0.88	2.75 ± 0.86	2.61 ± 1.00	0.071
Apo A1 (mmol/L)	1.06 (0.97-1.19)	1.07 (0.97-1.20)	1.05 (0.94-1.19)	0.115
Apo B (mmol/L)	0.94 (0.75-1.09)	0.94 (0.75-1.09)	0.93 (0.77-1.07)	0.885
TG/HDL-C (mmol/mmol)	1.44 (0.88-2.72)	1.43 (0.86-2.72)	1.59 (0.94-2.82)	0.239
LDL-C/Apo B (mmol/mmol)	2.88 (2.56-3.3)	2.91 (2.59-3.34)	2.74 (2.38-3.08)	<0.001
HDL-C/Apo A1 (mmol/mmol)	1.03 (0.93-1.13)	1.03 (0.93-1.13)	1.02 (0.92-1.12)	0.909
Apo B/Apo A1 (mmol/mmol)	0.88 ± 0.27	0.87 ± 0.27	0.90 ± 0.29	0.204

Normally distributed values in the table are given as the mean ± SD, skewed distributed values are given as the median (25 and 75% interquartiles), and categorical variables are given as frequency (percentage).

T2D, type 2 diabetes; DKD, diabetic kidney disease; BMI, body mass index; SBP/DBP, systolic/diastolic blood pressure; DPP-4 inhibitors, dipeptidyl peptidase-4 inhibitors; SGLT-2 inhibitors, sodium-glucose co-transporter-2 inhibitors; CCB, calcium channel blockers; ARB, angiotensin receptor blockers; HbA1c, glycosylated hemoglobin A1c; BUN, blood urea nitrogen; Cr, creatinine; Serum UA, serum uric acid; eGFR, estimated glomerular filtration rate; UACR, urine albumin/creatinine ratio; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B.

Hyperlipidemia is a well-recognized risk factor for DKD in patients with T2D. Hyperlipidemia can facilitate apoptosis of podocytes, a specialized kidney epithelial cell, thus damaging the integrity of the glomerular filtration barrier and eventually causing the onset of proteinuria (16). In addition to podocytes, hyperlipidemia can also promote glomerulosclerosis, interstitial fibrosis and accelerate the progression of proteinuria by affecting glomerular endothelial cells and mesangial cells and promoting the accumulation of collagen and fibronectin (17). Renal tubular injury is a vital component of DKD, and even precedes the occurrence of glomerular injury (18). Hyperlipidemia can cause renal tubular injury when combined with proteinuria by the mechanism that albumin can act as a carrier of fatty acids and promote the deposition of fatty acids in kidney (19). Additionally, ectopic deposition of lipids in kidney can induce local oxidative stress and inflammation *via* generating excess adipokines and activating a variety of signaling pathways (20). The present study demonstrated that the LDL-C/Apo B ratio was closely related to the prevalence of DKD in patients with T2D.

There have been many studies focused on investigating the relationships between lipid indices and DKD, but there are discrepancies among these studies. In a 2.9 years follow-up study, high levels of TG and low levels of HDL-C at baseline, but not levels of LDL-C could predict the decline of renal function (21). Slightly different from the above study, Retnakaran R et al. showed that serum TG and LDL-C levels were predictors of proteinuria in

TABLE 2 | Relationships between lipid indices and kidney damage indices in patients with T2D.

Lipid indices	Cystatin C		UACR		eGFR	
	r	*p* value	*r*	*p* value	*r*	*p* value
TG	-0.150	0.668	0.127	<0.001	0.046	0.190
TC	-0.130	0.210	0.034	0.311	0.119	0.245
HDL-C	-0.102	0.004	-0.027	0.420	0.026	0.454
LDL-C	0.074	0.028	-0.032	0.347	-0.077	0.329
Apo A1	-0.043	0.227	0.015	0.669	0.025	0.483
Apo B	0.084	0.017	0.050	0.143	-0.013	0.718
TG/HDL-C	0.017	0.621	0.123	<0.001	0.031	0.376
LDL-C/Apo B	-0.237	<0.001	-0.120	<0.001	0.146	<0.001
HDL-C/Apo A1	-0.129	<0.001	-0.045	0.191	0.051	0.152
Apo B/Apo A1	0.116	0.001	0.031	0.362	-0.039	0.267

r spearman's correlation coefficient

T2D, type 2 diabetes; UACR, urine albumin/creatinine ratio; eGFR, estimated glomerular filtration rate; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B.

TABLE 3 | Multivariate logistic regression analysis to identify the association of LDL-C/Apo B ratio with DKD.

Models	*B*	SE	Wald	*p*	OR	95% CI
Model 0	-0.580	0.160	13.193	<0.001	0.560	0.409-0.766
Model 1	-0.620	0.205	9.124	0.003	0.538	0.360-0.804
Model 2	-0.731	0.286	6.546	0.011	0.481	0.275-0.843

Model 0: unadjusted model.

Model 1: adjusted for age, male, diabetic duration, smoking history, BMI, hypertension, SBP, DBP.

Model 2: additionally adjusted for HbA1c, antidiabetic treatments, antihypertensive treatments, statins medications.

TABLE 4 | ORs (95% CIs) of DKD according to the four subgroups.

Variable	Group A	Group B	Group C	Group D	*P* value
LDL-C/Apo B ratio range	2.881-7.387	0.732-2.879	2.881-7.387	0.732-2.879	–
Number	289	240	179	228	–
DKD	25 (8.7)	51 (21.3)	23 (12.8)	45 (19.7)	<0.001
Model 0	1-reference	2.850 (1.705-4.763)	1.557 (0.855-2.837)	2.597 (1.538-4.385)	<0.001
Model 1	1-reference	2.190 (1.167-4.113)	2.235 (1.066-4.684)	3.743 (1.955-7.167)	0.001
Model 2	1-reference	2.205 (1.136-4.280)	2.315 (1.078-4.974)	3.513 (1.762-7.004)	0.005

Group A: normal lipid profile with a high LDL-C/Apo B ratio (> 2.880); Group B: normal lipid profile with a low LDL-C/Apo B ratio (< 2.880); Group C: abnormal lipid profile with a high LDL-C/Apo B ratio (> 2.880); Group D: abnormal lipid profile with a low LDL-C/Apo B ratio (< 2.880).

Model 0: unadjusted model.

Model 1: adjusted for age, male, diabetic duration, smoking history, BMI, hypertension, SBP, DBP.

Model 2: additionally adjusted for HbA1c, antidiabetic treatments, antihypertensive treatments, statins medications.

patients with T2D (22). In a longitudinal study involving a large Japanese population, the TG/HDL-C ratio was positively associated with the increase of proteinuria and the decline of eGFR (23). In contrast, no associations were observed between lipid indices and DKD or DKD markers in other studies (24–26). UACR, eGFR and cystatin C, respectively reflecting glomerular filtration barrier, glomerular filtration function and tubular function, all are important indicators of kidney function (27). In this study, we observed that both TG and TG/HDL-C ratio were only positively associated with UACR, while Apo B/Apo A1 ratio was only positively associated with cystatin C, which indicated that these lipid indices were insufficient to comprehensively assess renal injury in patients with T2D. These inconsistencies among these studies and our study may be attributed to differences in the included population, uses of statin. In this study, the LDL-C/Apo B ratio was significantly correlated with kidney injury indices, suggesting that LDL-C/Apo B ratio was most closely correlated with DKD among multiple lipid indices.

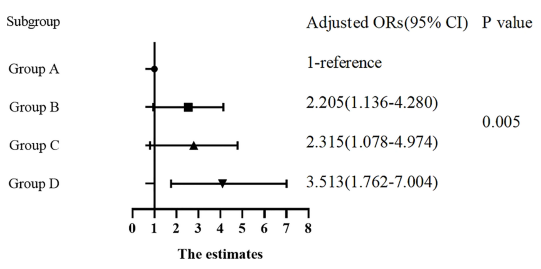

FIGURE 1 | Forest plot visualizing adjusted ORs based on logistic regression analysis.

A review published in 2020 suggested that hyperlipidemia may promote atherosclerosis independent of LDL-C concentration, which may be partly attributed to the presence of small dense LDL-C (sd-LDL-C) (28). LDL-C is a group of apolipoproteins with greater heterogeneity, and LDL-C with small particle size and high density is named as sd-LDL-C (29). Methods such as nuclear magnetic resonance and hypervelocity centrifugation can be used to evaluate sd-LDL-C, but these methods are not suitable for clinical practice due to high costs and lengthy processes (30). The LDL-C/Apo B ratio widely used in clinical and research studies has been identified as a surrogate marker for sd-LDL-C, and the smaller the LDL-C/Apo B ratio, the greater the proportion of sd-LDL-C (31). The present study found that even in patients with normal lipid profiles, having a low LDL-C/Apo B ratio significantly increased the risk of DKD in patients with T2D.

Compared with other LDL-Cs, less sd-LDL-Cs binding to LDL-C receptors result in sd-LDL-C staying in the circulation longer (32), and sd-LDL-Cs tend to be oxidized and modified to form oxidized LDL (33). Oxidized LDL, a marker of endothelial dysfunction and oxidative stress, can be excessively uptaken by multiple types of kidney cells, thus promoting glomerulosclerosis and kidney fibrosis, eventually accelerating the process of DKD (34). In addition, macrophages can be attracted by oxidized LDL, chemotaxis and phagocytosis oxidized LDL to form foam cells. Subsequently, the accumulation of foam cells in renal arterioles aggravates local renal hemodynamic disorders (35). Sodium-glucose co-transporter-2 inhibitors are a novel class of hypoglycemic agents recommended for type 2 diabetic patients with DKD by a group of guidelines, and a clinical study showed that the use of SGLT2 inhibitors could significantly reduce the level of sd-LDL-C in patients with T2D (36). These results together suggested that a low LDL-C/Apo B ratio was an important risk factor and a potential target for DKD in patients with T2D.

This study had several limitations. First, as this study was a cross-sectional study, the causal relationship between the LDL-C/Apo B ratio and DKD could not be fully elucidated. Second, the LDL-C/Apo B ratio was a proxy for evaluating sd-LDL-C rather than the gold standard, but the large sample size of this study compensated for this deficiency. Third, the generalizability of this study was limited by that this study was based on a Chinese population. Therefore, longitudinal and intervention studies are needed to address the above limitations.

In summary, the LDL-C/Apo B ratio was closely associated with the risk of DKD, and a lower LDL-C/Apo B ratio might be a potent risk factor and therapeutic target for prevention and treatment of DKD in patients with T2D. Even in type 2 diabetic patients with normal lipid profile, the LDL-C/Apo B ratio should be routinely assessed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The medical research ethics committee of Second Affiliated Hospital of Nantong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-FL and Z-HC participated in the design of the study, data collection, analysis of the data, and drafting of the manuscript. X-QW and H-YH conceived of the study, participated in its design and revised the manuscript. W-SL and L-YH participated in data collection. All authors read and approved the final manuscript.

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REG1A and RUNX3 Are Potential Biomarkers for Predicting the Risk of Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease. Clinical features are traditionally used to predict DKD, yet with low diagnostic efficacy. Most of the recent biomarkers used to predict DKD are based on transcriptomics and metabolomics; however, they also should be used in combination with many other predictive indicators. The purpose of this study was thus to identify a simplified class of blood biomarkers capable of predicting the risk of developing DKD. The Gene Expression Omnibus database was screened for DKD biomarkers, and differentially expressed genes (DEGs) in human blood and kidney were identified *via* gene expression analysis and the Least Absolute Shrinkage and Selection Operator regression. A comparison of the area under the curve (AUC) profiles on multiple receiver operating characteristic curves of the DEGs in DKD and other renal diseases revealed that *REG1A* and *RUNX3* had the highest specificity for DKD diagnosis. The AUCs of the combined expression of *REG1A* and *RUNX3* in kidney (AUC = 0.929) and blood samples (AUC = 0.917) of DKD patients were similar to each other. The AUC of blood samples from DKD patients and healthy individuals obtained for external validation further demonstrated that *REG1A* combined with *RUNX3* had significant diagnostic efficacy (AUC=0.948). *REG1A* and *RUNX3* expression levels were found to be positively and negatively correlated with urinary albumin creatinine ratio and estimated glomerular filtration rate, respectively. Kaplan-Meier curves also revealed the potential of *REG1A* and *RUNX3* for predicting the risk of DKD. In conclusion, *REG1A* and *RUNX3* may serve as biomarkers for predicting the risk of developing DKD.

Keywords: diabetic kidney disease, biomarkers, diagnosis, gene expression omnibus, disease risk prediction

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INTRODUCTION

With the increasing incidence of diabetes mellitus (DM) worldwide, diabetic kidney disease (DKD) has become the leading cause of end-stage renal disease with a high mortality rate (1). Approximately one-quarter of people with DM end up developing DKD (2). DKD diagnosis is primarily based on renal pathology and/or clinical manifestations and involves the determination of glomerular filtration rate (eGFR) and urinary protein levels in addition to clinical features such as the duration of DM and the presence of diabetic retinopathy (3, 4). However, eGFR and albuminuria are deficient in terms of sensitivity and specificity, while renal puncture is not widely available due to its invasiveness. Therefore, markers associated with DKD risk need to be identified for successful diagnosis and prevention. A meta-analysis showed that urinary transferrin excretion rate was a good predictor of DKD occurrence (5). Kidney injury molecule 1 is a membrane protein expressed in the apical membrane of proximal tubule cells and is of great value in assessing the progression of DKD (6). After 12 years of follow-up in a cohort study of 628 patients with DM, plasma tumor necrosis factor levels were found to be associated with early decline in eGFR was strongly correlated (7). Older age, male sex, prolonged diabetes duration, hypertension, glycated hemoglobin levels (HbA_{1c}), and plasma triglyceride levels were identified as risk factors for proteinuria in patients with type 2 DM in a previous meta-analysis of 13 studies (8). Several biomarkers to assess DKD risk have been identified as a result of recent advancements in analytical techniques as well. Mayer et al. also identified nine serum markers associated with decreased GFR in Type 2 DM, including chitinase 3-like protein 1, growth hormone 1, hepatocyte growth factor, matrix metalloproteinase 2 (MMP2), MMP7, MMP8, MMP13, tyrosine kinase, and tumor necrosis factor receptor 1 (9). The surrogate markers for micro- and macro-vascular hard endpoints for innovative diabetes tools study further identified five serum markers (fibroblast growth factor 21, symmetric-to-asymmetric dimethylarginine ratio, β2-microglobulin, C16-acylcarnitine, and kidney injury molecule 1) with significant prediction potential for GFR decline in patients with type 2 DM (10). Although traditional clinical characteristics are easily determined, their prognostic value is limited. In general, serum biomarkers have a higher predictive efficacy than clinical characteristics; however, multiple biomarkers should be used in combination to achieve such efficacy. Simple biomarker combinations often lack specificity. Thus, widespread application of serum biomarkers is hindered.

Genome-wide transcriptome analysis has been widely used in the field of DKD to help gain insight into disease pathogenesis, molecular classification and identification of biomarkers (11). These gene expression matrices are included in the Gene Expression Omnibus (GEO) database to facilitate integration and reanalysis by researchers. The goal of this study was to establish a simplified class of blood biomarkers for predicting the risk of DKD. The study flow is illustrated in **Figure 1**.

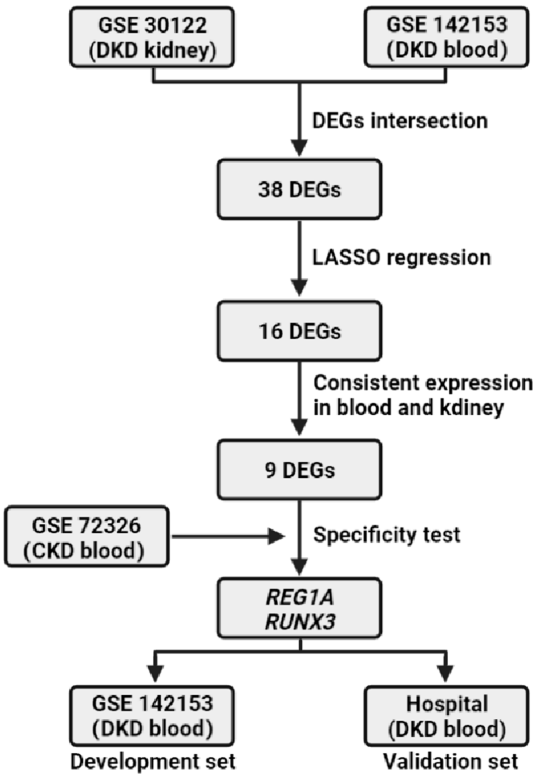

FIGURE 1 | Flowchart. DKD, diabetic kidney disease; CKD, Chronic kidney disease.

METHOD

Data Acquisition

We acquired gene expression profile datasets GSE30122 (11), GSE142153 (12), and GSE72326 (13) from the GEO database (14) (https://www.ncbi.nlm.nih.gov/geo/), and performed gene ID and symbol conversions *via* Perl scripts (15). GSE30122 included kidney tissue from patients with pathologically confirmed DKD stage 4-5, with healthy kidney tissue as a control. GSE142153 incorporates peripheral blood from patients with a clinical diagnosis of DKD stages 3-5, with healthy human blood as a control. GSE142153 included peripheral blood from patients with confirmed CKD, with blood from people without kidney disease as a control.

Identification of Differentially Expressed Genes

R software (version 4.1.0, http://r-project.org/) was used for data analysis and plotting. The "limma" R package (16) was used to screen for DEGs, and heatmap and volcano plots of DEGs were constructed by the "ggplot2" package (17) to visualize the expression levels of DEGs. $p < 0.05$ was considered statistically significant.

Screening and Validation of Diagnostic Markers

The Least Absolute Shrinkage and Selection Operator (LASSO) method, as implemented in the "glmnet" software package, was used for reduction of data dimensionality and selection of predictive features for DKD patients (18, 19). The receiver operating characteristic (ROC) curve method is used for evaluation of diagnostic performance (20). The area under the curve (AUC) of ROCs of single or multiple factors was calculated using the " pROC" software package (21). Calibration curves were plotted using the "rms" software package to assess whether the predicted probability of the model approximated the true probability (22, 23).

Correlation of Diagnostic Markers With Clinical Characteristics

The "ggstatsplot" package was used to perform Spearman correlation analysis of diagnostic markers and clinical features (24), and the results were subsequently visualized using the "ggplot2" package.

Analysis of the Prognostic Potential of Identified Biomarkers

Kaplan-Meier (KM) curves were constructed using "survival" and "survminer" software packages to assess the probability of DKD occurring at specific time periods, and log-rank tests were used to determine differences between groups (25). The prognostic value of the diagnostic markers was assessed using univariate or multivariate Cox proportional hazards models.

Clinical Statistics

A total of 141 human blood samples from patients with DKD, DM (without DKD), and healthy individuals, were collected from the biospecimen bank of Shenzhen People's Hospital. The study was approved by the Ethics Committee of Shenzhen People's Hospital. Data are expressed as mean plus/minus standard deviation (SD) or median and interquartile range for continuous variables and as percentages for categorical variables. The Mann-Whitney U-test or t-test was performed to compare the differences between the two groups depending on whether the data conformed to a normal distribution. Chi-square test was applied to compare frequencies. This work was approved by the Institutional Review Board and the Ethics Committee of the Shenzhen People's Hospital (No. LL-KT-2018338) and informed written consent was obtained from all participants.

Quantitative Real-Time PCR Analysis

Trizol (Invitrogen) was used to extract total RNA from peripheral blood mononuclear cells (PBMC) according to manufacturer's instructions. Reverse transcription of RNA was performed using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR was performed using PowerUp SYBR Green Master Mix (Thermo Scientific). The results were standardized using GAPDH. qPCR was conducted using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Fold-change was determined as $2^{-\triangle\triangle Ct}$ for gene expression. Gene-specific PCR primers are listed in **Table 1**.

RESULTS

Screening for DEGs Shared Across Blood and Kidney Samples

Figure 2 shows the screening process for DEGs shared across blood and kidney biopsy samples from patients with DKD. Using the healthy control samples (HC) as basis, 679 (GSE142153: HC, n=10, DKD, n=23) and 499 (GSE30122: HC, n=50, DKD, n=19) DEGs were identified in blood and kidney samples from patients with DKD, respectively. A total of 38 DEGs were found to be shared across blood and kidney biopsy samples from patients with DKD.

Screening for DEGs With Consistent Expression in DKD Blood and Kidney Samples

The LASSO logistic regression algorithm was used to further screen DEGs shared across blood samples from patients with DKD (GSE142153). A total of 16 DEGs were identified as diagnostic DKD markers (**Figures 3A, B**), including 5 and 11 down- and up-regulated genes, respectively (**Figure 3C**). Two of these DEGs were down-regulated whereas 14 were upregulated in kidney biopsy samples from patients with DKD (GSE30122) (**Figure 3D**). Ultimately, nine DEGs with consistent expression profiles in blood and kidney biopsy samples from patients with DKD were identified: which included Alpha-2A adrenergic receptor (*ADRA2A*), C-C motif chemokine 5 (*CCL5*), cholesterol 25-hydroxylase (*CH25H*), C-X-C chemokine receptor type 4 (*CXCR4*), hemoglobin subunit delta (*HBD*), hydroxycarboxylic acid receptor 3 (*HCAR3*), lysophosphatidylcholine acyltransferase 1 (*LPCAT1*), Lithostathine-1-alpha (*REG1A*), and Runt-related transcription factor 3 (*RUNX3*). The distribution of the expression profiles of these DEGs in the blood and kidney of DKD patients (**Figures 3E, F**) revealed that expressions of all of them were upregulated.

Screening for Diagnostic DEGs in DKD

Two blood transcriptome datasets were analyzed to determine DKD-specific DEGs for diagnosis. In the GSE142153 dataset, several DEGs (*ADRA2A*, *CCL5*, *CH25H*, *CXCR4*, *HBD*, *HCAR3*, *LPCAT1*, *REG1A*, and *RUNX3*) were found to have high diagnostic efficiency in blood samples (AUC > 0.70), except for *CH25H* (AUC =0.67) (**Figure 4A**). The GSE72326 dataset [lupus nephritis (LN), n=48; ANCA-associated nephritis, n=10; focal segmental sclerosis (FSGS), n = 3; IgA nephropathy (IgAN), n = 5; microscopic disease (MCD), n =3] was used to validate the specificity of these DEGs for DKD diagnosis, except for *HCAR3*, which was excluded from the analysis as it was not found in the dataset. The diagnostic efficacies of eight DEGs in patients with LN were found to be relatively low (**Figure 4B**). *CXCR4*, *LPCAT1*, and *HBD* showed high diagnostic efficacy for ANCA-associated nephritis (**Figure 4C**). Significant diagnostic performance of *CXCR4*, *CCL5*, and *HBD* was observed for

TABLE 1 | The sequences of primers for qRT-PCR analysis.

Gene	Forward	Backward
***Homo RUNX3* (Gene ID: 864)**	AGGCAATGACGAGAACTACTCC	CGAAGGTCGTTGAACCTGG
Homo *REG1A* (Gene ID: 5967)	ACCAGCTCATACTTCATGCTGA	CCAGGTCTCACGGTCTTCAT
Homo *GAPDH* (Gene ID: 2597)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

patients with FSGS (**Figure 4D**). *ADRA2A*, *LPCAT1*, and *HBD* showed high diagnostic efficacy for patients with IgAN (**Figure 4E**). *CH25H* and *HBD* showed good diagnostic efficacy in patients with MCD (**Figure 4F**). In summary, the transcription level of *REG1A* and *RUNX3* in blood samples may serve as DKD-specific predictors of disease development.

DKD Diagnosis Efficacy of *RUNX3* and *REG1A* in Blood and Kidney Samples

Expression levels of *REG1A* and *RUNX3* were found to be significantly increased in blood samples of patients with DKD (**Figures 5A, B**; GSE142153). The combined diagnostic efficacy of these genes was also high (AUC=0.917, 95% CI: 0.818-1) (**Figure 5C**). Similar results have been obtained from kidney samples from DKD patients as well (**Figures 5D, E**; GSE30122, AUC=0.929, 95% CI: 0.846-1, **Figure 5F**). These findings indicate that *REG1A* and *RUNX3* have diagnostic potential for DKD in blood as well as kidney.

Diagnostic Performance of *REG1A* and *RUNX3* in the Validation Set

A total of 141 blood samples from the human biospecimen bank of Shenzhen People's Hospital were used for qPCR analysis of *REG1A* and *RUNX3*. DKD (n = 50) and HC (n = 41) groups were included in the validation set (**Table 2**). The expression of *REG1A* and *RUNX3* were found to be significantly upregulated in the DKD group compared to those in the HC group (**Figures 6A, B**). The AUC for *REG1A* and *RUNX3* were found to be 0.912 and 0.859, respectively (**Figure 6C**). When *REG1A* and *RUNX3* were fitted as a single variable, the diagnostic efficiency was found to be 0.917 (**Figure 6C**) for the development set, and even higher for the validation set (AUC=0.948, 95% CI: 0.898-0.998) (**Figure 6D**), indicating that *REG1A* and *RUNX3* have high diagnostic value. A high degree of agreement was also found between the predicted and true values of the calibration curves in both development and validation sets (**Figures 6E, F**), indicating significant efficacy of *REG1A* and *RUNX3* for predicting DKD development.

Analysis of Correlation Between Identified DEGs and Clinical Characteristics

The DKD (n=50) and DM (without DKD) groups (n=50) in the validation set were used to analyze correlation between expression levels of diagnostic DEGs and clinical characteristics (**Table 3**). Expression levels of *REG1A* and *RUNX3* were found to be significantly higher in the DKD group than those in the DM group (**Figures 7A, B**). *REG1A* was found to be positively correlated with serum creatinine (SCr), C-peptide (C-P), HbA_{1C}, fasting blood glucose (FBG), and urinary albumin creatinine ratio (UACR) and negatively correlated with eGFR level (**Figure 7C**, **Supplementary Figure 1**). *RUNX3* was positively correlated with UACR and SCr and negatively correlated with eGFR level (**Figure 7D**, **Supplementary Figure 2**). *REG1A* and *RUNX3* expression levels were found to be positively correlated as well (r=0.3) (**Supplementary Figure 1G**).

KM Analysis of Diagnostic Markers and Clinical Characteristics

The KM method was used to analyze the probability of DKD occurrence in the corresponding time periods, and the variables included diagnostic DEGs and clinical characteristics. Using

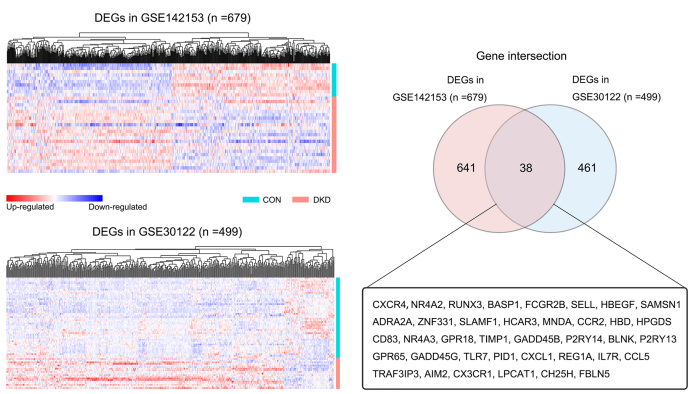

FIGURE 2 | Screening of blood and kidney co-DEGs. Heat map of all DEGs in the blood (GSE142153) and kidney datasets (GSE30122) (DKD vs. HC, $p < 0.05$). The Venn diagram shows that there are 38 DEGs shared across blood and kidney samples.

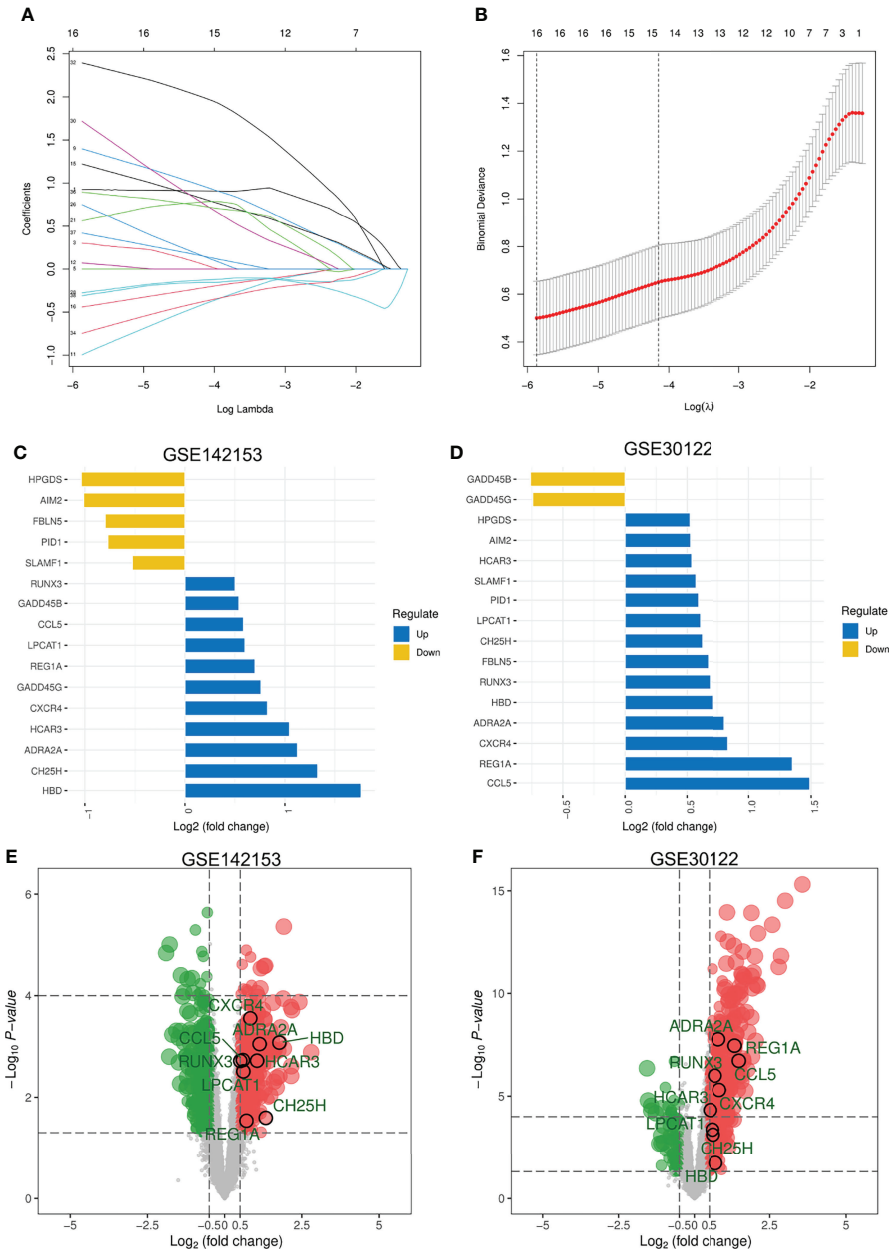

FIGURE 3 | Screening for DEGs with Consistent Expression in DKD Blood and Kidney Samples. **(A)** LASSO coefficient profiles of the 38 features. A coefficient profile plot was produced against the log(λ) sequence. A vertical line was drawn at the value selected using five-fold cross-validation, where the optimal lambda resulted in 16 features with nonzero coefficients. **(B)** Optimal parameter (λ) selection in the LASSO model used five-fold cross-validation *via* minimum criteria. The partial likelihood deviance (binomial deviance) curve was plotted versus log(λ). Dotted vertical lines were drawn at the optimal values by using the minimum criteria and the 1 SE of the minimum criteria (the 1-SE criteria). **(C, D)** Expression of 16 DEGs in blood and kidney. Five DEGs that were consistently expressed in blood and kidney. **(E)** Distribution of these 5 DEGs in DKD blood samples. **(F)** Distribution of the 5 DEGs in the kidney of DKD. LASSO, least absolute shrinkage and selection operator; SE, standard error.

DKD as the endpoint event, variables associated with negative prognosis included *REG1A*, *RUNX3*, total cholesterol (TC), FBG, SCr, body mass index (BMI), and UACR, whereas elevated levels of high-density lipoprotein cholesterol (HDL-C), age, and eGFR indicated a positive prognosis (**Table 4** and **Supplementary Figure 3**). Combined analysis showed that patients with high expression of *REG1A* and *RUNX3* had the worst prognosis in all four groups (HR = 6.459) (**Figure 8**).

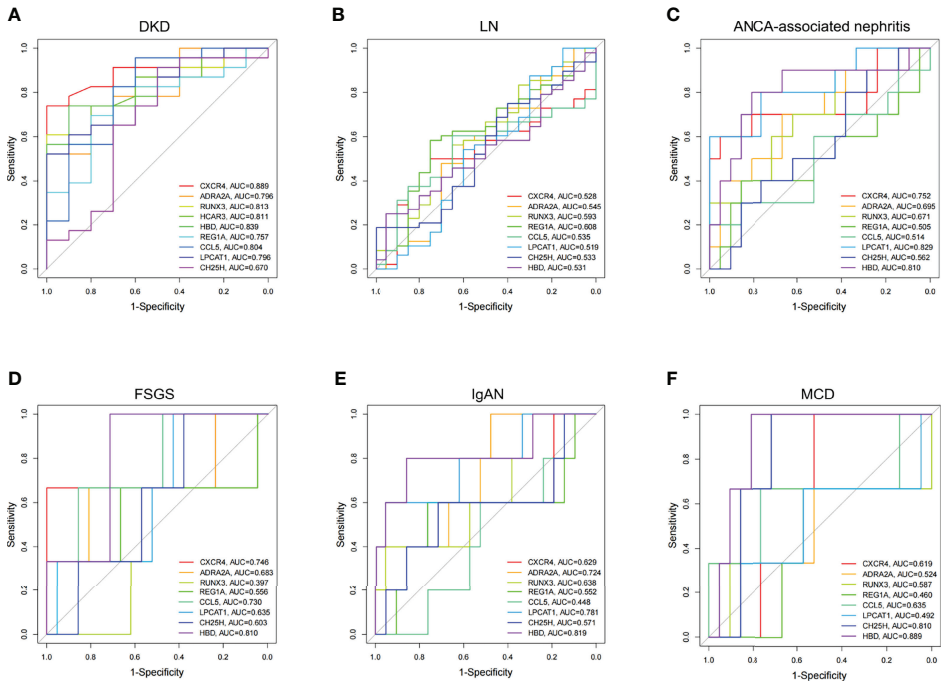

FIGURE 4 | Diagnostic efficacy of DEGs expressed consistently between blood and kidney in CKD. **(A)** Multivariate ROC curve of DEGs in DKD (GSE142153). **(B–F)** Multivariate ROC curve of DEGs in LN, ANCA-associated nephritis, FSGS, IgAN, and MCD, respectively (GSE72326). LN, lupus nephritis; ANCA, Anti-Neutrophil Cytoplasmic Antibodies; FSGS, focal segmental sclerosis; IgAN, IgA nephropathy; MCD, microscopic disease.

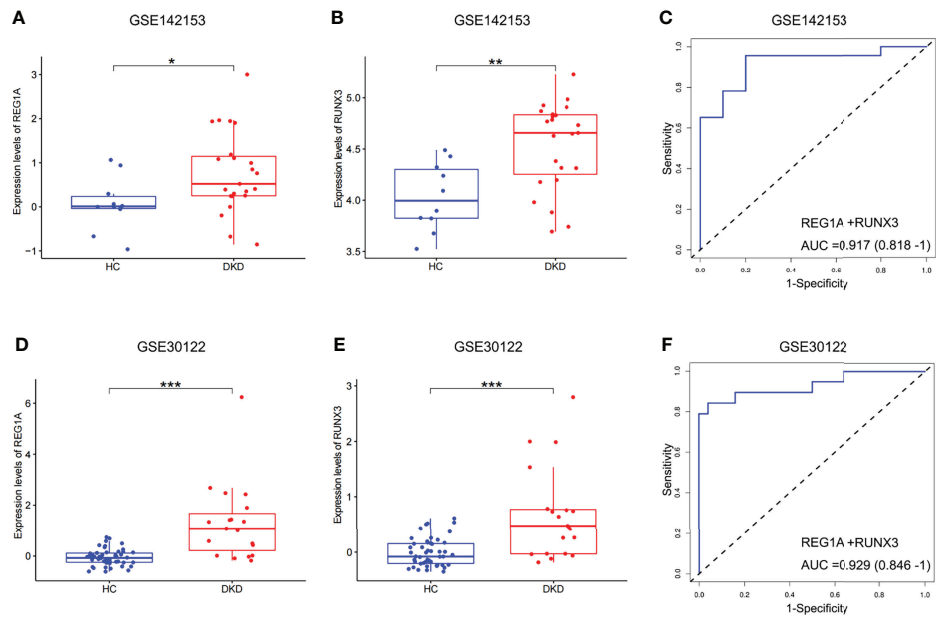

FIGURE 5 | Expression and validation of diagnostic marker efficacy in developmental cohorts. **(A, B)** Box plots showed that the expression levels of *REG1A* and *RUNX3* were significantly higher in blood samples (DKD vs. HC, rank sum test). **(C)** ROC curve of the combined diagnostic efficacy of *REG1A* and *RUNX3* in blood samples. **(D, E)** Box plots showed that the expression levels of *REG1A* and *RUNX3* were significantly higher in the kidney samples (DKD vs. HC). **(F)** ROC curve of the combined diagnostic efficacy of *REG1A* and *RUNX3* in kidney samples. *p <0.05, **p <0.01, ***P <0.001.

TABLE 2 | Baseline information on diagnostic markers and clinical characteristics in the validation cohort.

	Overall	HC	DKD	p
n	91	41	50	
Female (%)	32 (35.2)	17 (41.5)	15 (30.0)	0.358
Age (years)	56.00 [52.00, 66.00]	54.00 [52.00, 61.00]	57.00 [52.00, 67.00]	0.211
RUNX3	1.49 [1.00, 2.82]	1.07 [0.74, 1.38]	2.63 [1.58, 3.66]	<0.001
REG1A	1.68 [0.94, 2.98]	0.96 [0.84, 1.09]	2.84 [2.02, 3.82]	<0.001

Data are shown as mean (SD), median [25% quartile, 75% quartile] or numbers (percentages).

DISCUSSION

In the present study, we found that serum RUNX3 and REG1A have potential as diagnostic markers for DKD. Patients with DM who have elevated expression of both RUNX3 and REG1A will have a much higher risk of developing DKD at about 10 years into the disease. Compared to existing studies of diagnostic markers for DKD (26, 27), we have achieved high diagnostic efficacy with the combination of only two markers. Importantly, the diagnostic efficacy of RUNX3 and REG1A in blood is not inferior to that of renal tissue, which facilitates their widespread use. RUNX3 and REG1A are more specific in the diagnosis of

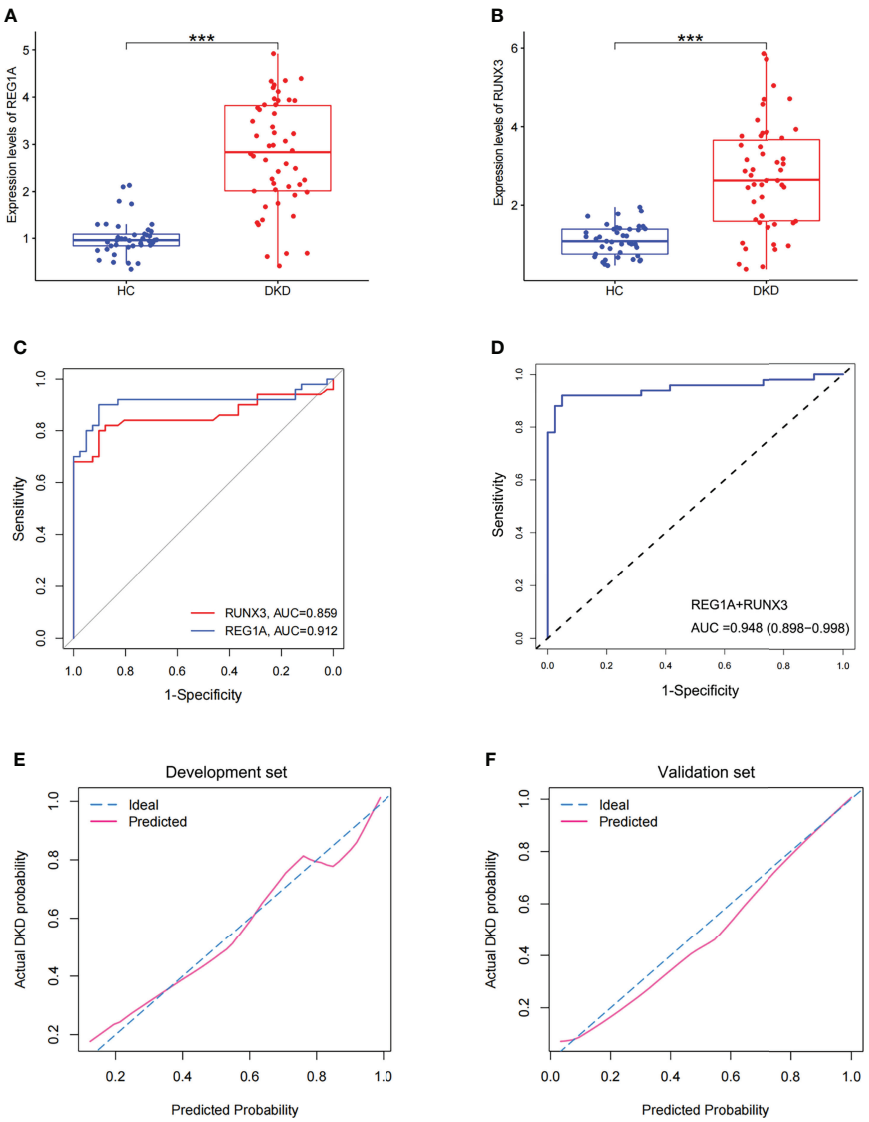

FIGURE 6 | Expression and diagnostic efficacy of diagnostic markers in the validation cohort. **(A, B)** Box plots showed significantly higher expression levels of *REG1A* and *RUNX3* (DKD vs. HC). **(C)** Separate ROC curves of *REG1A* and *RUNX3*. **(D)** The ROC curve of the diagnostic efficacy verification after fitting two diagnostic markers to one variable. **(E, F)** Calibration curves of diagnostic markers. The diagonal dotted line represents a perfect prediction by an ideal model. The solid line represents the performance of the nomogram, of which a closer fit to the diagonal dotted line represents a better prediction. The fit of both the dashed and solid lines for the development set GSE142153 **(E)** and the validation set **(F)** was excellent. ***P <0.001.

TABLE 3 | Baseline information on diagnostic markers and clinical characteristics in the DM and DKD groups.

	Overall	DM	DKD	p
n	100	50	50	
Female (%)	30 (30.0)	15 (30.0)	15 (30.0)	1
Age (years)	57.65 (10.49)	56.40 (9.63)	58.90 (11.24)	0.235
Duration of DM (years)	12.26 (6.59)	10.59 (5.53)	13.56 (7.10)	0.016
BMI (kg/cm^2)	24.10 (3.15)	24.13 (3.15)	24.06 (3.18)	0.916
FBG (mmol/L)	6.84 [5.54, 8.51]	6.68 [5.61, 8.44]	7.36 [5.46, 8.48]	0.743
C-P (ng/ml)	1.65 [0.89, 2.51]	1.65 [1.24, 2.30]	1.58 [0.67, 3.07]	0.986
HbA1c (%)	8.89 (2.09)	8.54 (1.96)	9.25 (2.17)	0.092
SCr (μmol/L)	80.50 [65.00, 131.25]	69.50 [60.25, 79.50]	131.50 [86.75, 181.00]	<0.001
eGFR (ml/min/1.73 m^2)	84.21 [47.02, 101.26]	98.13 [88.74, 104.92]	46.30 [32.20, 80.08]	<0.001
UA (μmol/L)	375.22 (110.66)	343.68 (97.29)	406.76 (115.08)	0.004
UACR (mg/g)	120.12 [7.42, 1932.66]	7.40 [4.27, 16.40]	1951.62[1025.49, 2908.83]	<0.001
TG (mmol/L)	1.40 [0.97, 2.16]	1.23 [0.92, 2.13]	1.45 [1.10, 2.17]	0.201
TC (mmol/L)	4.53 [3.62, 5.72]	4.28 [3.63, 5.47]	4.85 [3.68, 6.11]	0.222
HDL-C (mmol/L)	1.07 [0.89, 1.21]	1.08 [0.93, 1.22]	1.05 [0.82, 1.21]	0.218
LDL-C (mmol/L)	2.56 [1.85, 3.73]	2.54 [1.88, 3.45]	2.63 [1.85, 3.77]	0.563
RUNX3	1.58 (1.07)	1.00 (0.62)	2.18 (1.10)	<0.001
REG1A	1.11 (0.47)	1.00 (0.41)	1.20 (0.49)	0.02

Data are shown as mean (SD), median [25% quartile, 75% quartile], or numbers (percentages). BMI, body mass index; FBG, fasting blood glucose; C-P, C-peptide; HbA1c, glycated hemoglobin A1c; SCr, serum creatinine; eGFR, estimation of glomerular filtration rate; UA, uric acid; UACR, urine albumin creatinine ratio; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoproteins cholesterol; LDL-C, low-density lipoproteins cholesterol.

DKD compared to markers such as urinary transferrin, urinary IgG and urinary type IV collagen (28).

RUNX transcription factors regulate various biological processes, including embryonic development, cell proliferation, differentiation, cell lineage determination, and apoptosis (29). *RUNX3* plays a downstream role in the TGF-β signalling pathway (30). Smyth et al. reported that *RUNX3* methylation is significantly increased in the blood of patients with DKD (31). In high glucose-treated renal tubular epithelial cells, RUNX3 was found to regulate the TGF-β1/Smad signalling pathway (32).

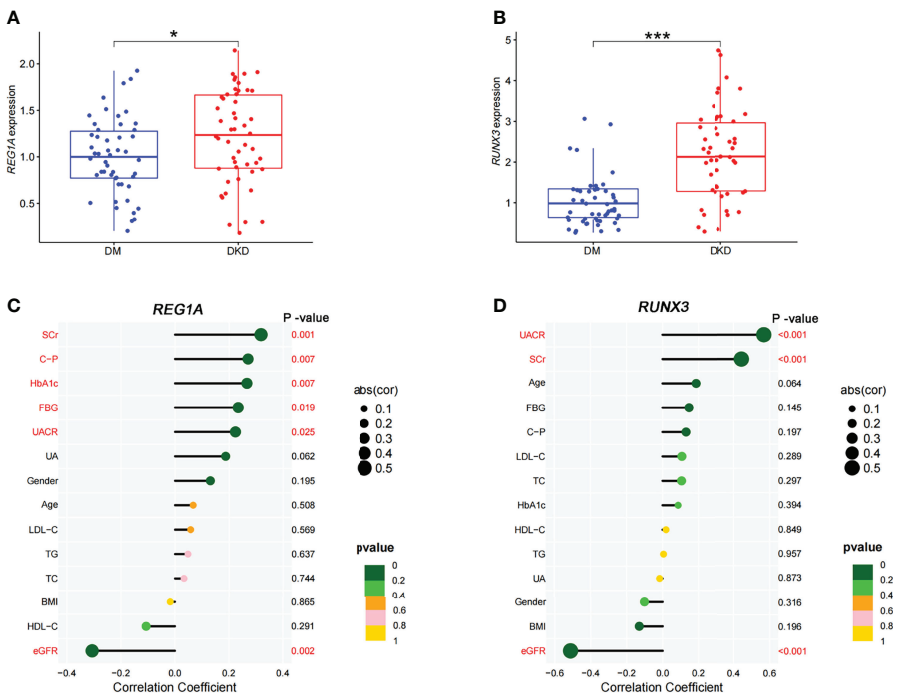

FIGURE 7 | Expression of diagnostic markers and their correlation with clinical characteristics in the DKD and DM cohort. **(A, B)** Box plots showed significantly higher expression levels of *REG1A* and *RUNX3* (DKD vs. DM). **(C)** Correlation of *REG1A* and clinical characteristics. **(D)** Correlation of *RUNX3* and clinical characteristics. The size of the dots represents the correlation between genes and clinical characteristics; the color of the dots represents the p-value. *p <0.05, ***P <0.001.

TABLE 4 | Univariate COX regression of diagnostic markers and clinical characteristics.

Variable	HR	lower 95%CI	upper 95%CI	p-value
RUNX3	2.50	1.43	4.36	<0.001
REG1A	2.97	1.35	4.17	0.003
TC	1.93	1.11	3.39	0.022
FBG	1.78	1.01	3.12	0.029
SCr	2.44	1.40	4.25	0.001
HDL-C	0.51	0.29	1.02	0.022
Age	0.4	0.22	0.75	<0.001
eGFR	0.34	0.19	0.61	0.002
BMI	1.84	1.02	3.31	0.019
UACR	5.62	3.22	9.81	<0.001

BMI, body mass index; FBG, fasting blood glucose; SCr, serum creatinine; eGFR, estimation of glomerular filtration rate; UACR, urine albumin creatinine ratio; TC, total cholesterol; HDL-C, high-density lipoproteins cholesterol. Showing variables with p-values <0.05.

Inhibition of *Runx3* expression ameliorates vascular endothelial dysfunction in DM mice (33). In diabetic patients, *RUNX3* was similarly found to be involved in diabetic endothelial progenitor cell dysfunction (34). High expression of *RUNX3* may exacerbate diabetic vascular endotheliopathy and ultimately lead to DKD. The REG family proteins are structurally similar to each other, and classified as calcium-dependent lectins (35). The mainstream view is that *REG1A* is an indicator of islet β-cell apoptosis and its elevation indicates a decline in islet function (36). In contrast, Okamoto et al. found that *REG1A* stimulates islet regeneration by inducing cell proliferation (37). Sobajima et al. reported significantly increased urinary *REG1A* levels in patients with DKD (38). Li et al. found that REG1A levels are significantly upregulated in the serum of patients with DKD, and possibly associated with renal injury as well (39). The above suggests that REG1A level is associated with impairment of islet function and DKD renal function. The above suggests that REG1A level is associated with islet and DKD renal injury. Our study also found that serum *REG1A* was significantly and positively associated with renal and islet impairment, while serum *RUNX3* was only associated with renal impairment. Our study also found that serum *REG1A* was significantly elevated in DM, whereas *RUNX3* was unchanged (**Supplementary Figure 4**). We therefore speculate that *REG1A* causes diabetes by destroying islet cells, whereas *RUNX3* causes DKD by directly destroying vascular endothelial cells.

To assess the risk of DKD development, we plotted KM curves for *REG1A*, *RUNX3* and the clinical characteristics. The results showed that people with high *REG1A* and *RUNX3* expression are at an increased risk of DKD after approximately 12 and 8 years of DM, respectively. Our findings are consistent with the results of most previous studies (40–44), which revealed incresaed TC, FBG, SCr, BMI, and UACR levels as risk factors for DKD, and increased eGFR and HDL-C levels are protective factors for DKD. Contrary to previous findings (45), aging was found to be a protective factor against DKD in this study. Afkarian et al. reported that youth-onset type 2 DM patients may have a higher risk of DKD (46). **Figure 8** shows the KM curves for different expression levels of *REG1A* and *RUNX3*. Accordingly, the risk of DKD was found to be highest when expression levels of both *REG1A* and *RUNX3* are high compared to other groups. Compared to patients with low expression of both *REG1A* and *RUNX3*, DM patients with high expression of both genes are at a rapidly increasing risk of developing DKD after 7-8 years of DM. Hence, *REG1A* and *RUNX3* are potential biomarkers for predicting the risk of developing DKD.

Limitation

In this study, when comparing the diagnostic efficacy of DEGs in chronic kidney disease (GSE72326), insufficient sample size may have led to unreliable results. We plan to continue collecting blood from patients with chronic kidney disease in subsequent

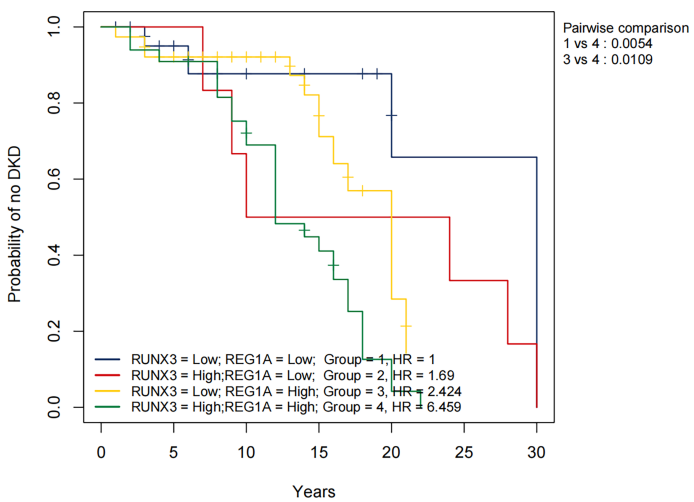

FIGURE 8 | Overall KM curves using combinations of *REG1A* and *RUNX3* expression levels. Univariate Cox regression was used to determine HR; log-rank p-values reported; Bonferroni multiple testing adjustment for pairwise comparisons. *REG1A* high and low cut-offs, 0.84; *RUNX3* high and low cut-offs 1.45. Time unit (years). HR, Hazard ratio.

studies to determine the specificity of *RUNX3* and *REG1A* in the diagnosis of DKD. We have so far only found elevated *RUNX3* and *REG1A* at the transcriptional level in the kidney and subsequent collection of kidney tissue from DKD patients for immunohistochemistry and protein immunoblotting is required.

Conclusion

REG1A and *RUNX3* were found to have high diagnostic efficacy for DK, which was proven through external validation. *REG1A* and *RUNX3* levels were positively and negatively correlated with UACR and eGFR levels, respectively. Thus, the transcription levels of *REG1A* and *RUNX3* in blood samples have potential to predict DKD risk.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Shenzhen people's hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

XW, HW and GY contributed equally to this work. XW, GY, HW and JX contributed to the acquisition of data, analysis and interpretation of data. SY and XW drafted the work or revised it critically for important intellectual content. LX, LZ, TL, and XZ, and LK analyzed the data and revised the article critically for important intellectual content. ZL and SY contributed to the conception and the study design. All authors gave their approval of the version to be published. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.935796/full#supplementary-material

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An overview of the efficacy and signaling pathways activated by stem cell-derived extracellular vesicles in diabetic kidney disease

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Diabetic kidney disease (DKD) is one of complications of diabetes mellitus with severe microvascular lesion and the most common cause of end-stage chronic kidney disease (ESRD). Controlling serum glucose remains the primary approach to preventing and slowing the progression of DKD. Despite considerable efforts to control diabetes, people with diabetes develop not only DKD but also ESRD. The pathogenesis of DKD is very complex, and current studies indicate that mesenchymal stromal cells (MSCs) regulate complex disease processes by promoting pro-regenerative mechanisms and inhibiting multiple pathogenic pathways. Extracellular vesicles (EVs) are products of MSCs. Current data indicate that MSC-EVs-based interventions not only protect renal cells, including renal tubular epithelial cells, podocytes and mesangial cells, but also improve renal function and reduce damage in diabetic animals. As an increasing number of clinical studies have confirmed, MSC-EVs may be an effective way to treat DKD. This review explores the potential efficacy and signaling pathways of MSC-EVs in the treatment of DKD.

KEYWORDS

diabetic kidney disease, mesenchymal stem cells, extracellular vesicles, signaling pathway, end-stage renal disease

Introduction

The global prevalence of diabetes is projected to rise from 9.3% in 2019 to 10.9% in 2045 (1). Diabetic kidney disease (DKD) is a serious complication caused by diabetes and occurs in 20-40% of people with diabetes (2, 3). Treatment of DKD has included blood pressure control with angiotensin receptor blockers or angiotensin-converting enzyme inhibitors and strict glycemic control (4). However, many patients still progress to the stage of end-stage renal disease (ESRD) (5). From 2000 to 2015, DKD as a percentage of chronic kidney disease increased from 22.1% to 31.3% (6) and is the most common cause of ESRD in many developed countries. When compared with other diabetic complications, the prevalence of DKD disease has not notably reduced over the past 20 years (7). There is an urgent need to develop effective therapeutic strategies to preserve the renal function and slow the progression of DKD.

Mesenchymal stromal cells (MSCs) are a group of cells with differentiation and proliferative potential (8). When treated with appropriate compounds, they can differentiate into cells of all mesodermal lineages, such as fibroblasts, myocytes, adipocytes, osteocytes, or chondrocytes (9, 10). Studies have shown that miR-124a can stimulate the differentiation of mesenchymal stem cells sourced bone marrow into islet-like cells and thus alleviate DKD (11). MSCs mainly used for DKD include adipose-derived (AD-MSCs), umbilical cord-derived (UC-MSCs), bone marrow-derived (BM-MSCs), and human urine-derived (HU-MSCs). MSCs protect the kidney in two ways. One is homing and differentiation, where MSCs recognize damaged tissue and then home and integrate into specific sites, and the other is through paracrine action. However, the main problem after direct administration of MSCs is that they do not target the target tissue. The infusion of MSCs *via* direct acute infusion or coupled with implanted continuous pumps directly into the kidney is the main way (12). After MSCs are injected into the tail vein of rats, most of the stem cells appear in the lungs (13). To determine whether MSCs exist in rat tissues, DNA was extracted from rat organs and a human Alu sequence was detected. Human Alu sequences were detected in the peritubular region, lung, and spleen in rats within 24 hours after injection of labeled MSCs, but rarely in the glomerulus and pancreas (14). Implanted continuous pumps directly into the kidney have the advantage of local tissue effect, but it is difficult to implement and difficult to popularize clinically. As only minimal numbers of donor MSCs are detected in the renal tissue, the therapeutic effect of MSCs on kidney injury appears to be attributable to lots of paracrine factors. These factors facilitate the renal repair by paracrine-mediated actions, including cell-cell interactions reactivating endogenous repair systems, and the release of extracellular vesicles (EVs) (15). This review explores the potential efficacy and signaling pathways of MSC-EVs in the treatment of DKD.

Characteristics of stem cell products

MSC-conditioned medium (MSC-CM) is rich in EVs secreted by MSCs. As stem cell products, EVs are generally classified into three categories: apoptotic bodies, microvesicles (MVs), and exosomes (Exos), which vary in size, origin, and release mechanism (16, 17). MVs are 50 nm-1000 nm in diameter and are shed directly from the cytoplasmic membrane (16, 18, 19). Their release is initiated by budding outward from the membrane surface (16, 19). Apoptotic bodies are released during apoptosis. The diameter of apoptotic vesicles is reported to range between 1000 nm and 5000 nm (20). The reported diameter of Exos is between 40 and 150 nm (21). Exos carry complex molecular cargoes such as proteins, lipids and nucleic acids (e.g., DNA, miRNA, circRNA) (Figure 1). A series of studies have shown that stem cell Exos protect the kidney from damage through multiple pathways involving anti-apoptotic, anti-inflammatory, anti-oxidative, anti-fibrotic roles and regulate podocyte autophagy (22, 23).

Amelioration of podocyte injury by stem cell products

Podocytes, important intrinsic cells of the glomerulus, are involved in protein filtration in the glomerulus and play an important role in the maintenance of kidney function (23, 24). Podocyte injury is an important characteristic of DKD. Podocyte loss contributes to the development of DKD (25). MSC-EVs are found that they can protect the podocytes in DKD in multiple ways, including inhibition of apoptosis and fibrosis and enhancement of autophagy, all perhaps due to the presence of a large number of growth factors and miRNAs in MSC-EVs (26).

High glucose (HG)-induced podocyte damage *in vitro* can mimic the state of podocytes in patients with DKD. Jiang et al. (27) conducted a study on human podocytes, and reported that HU-MSC-EVs reduced HG-induced podocyte apoptosis *in vitro*. UC-MSCs secrete EVs carrying bone morphogenetic protein-7 (BMP-7) and high levels of vascular endothelial growth factor (VEGF) (Table 1). The activation of cytokines such as BMP-7 or VEGF is important for the survival of podocyte. VEGFA is highly expressed in the podocytes and is required to maintain endothelial cell function (46). However, VEGFA is overexpressed in the early stage of DN, and blocking VEGFA reduces proteinuria in DN (47). Duan et al. (28) showed that silencing of VEGF attenuates podocyte inflammation and reduces apoptosis. Moreover, miR-16-5p encapsulated within HU-MSCs can inhibit VEGFA expression in podocytes induced by HG, promote podocyte viability and decrease the rate of apoptosis (Table 1). AD-MSC-EVs can transfer miR-26a-5p to

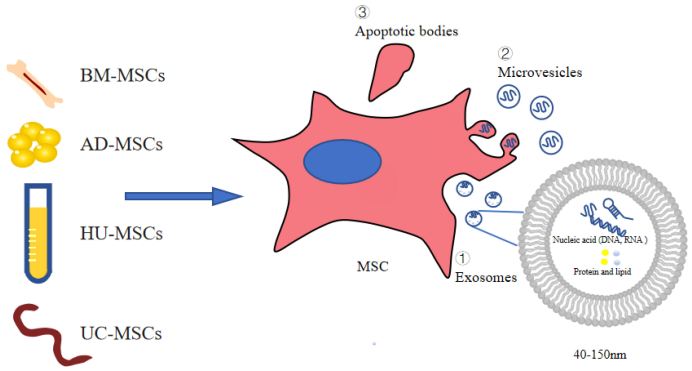

FIGURE 1
Extracellular vesicles derived from stem cells. AD-MSCs, adipose-derived MSCs; BM-MSCs, bone marrow-derived MSCs; UC-MSCs, umbilical cord-derived MSCs; HU-MSCs, human urine-derived MSCs; MSCs. mesenchymal stromal cells.

mouse podocytes *in vitro* to inhibit podocyte apoptosis by downregulating nuclear factor kappa-B (NF-κB)/VEGFA and toll-like receptor 4 (TLR4) signaling pathways (29). Pyruvate dehydrogenase kinase 4 (PDK4) is a molecular target of miR-15b-5p (48). Zhao et al. (30) reported that AD-MSC-CM miR-15b-5p directly binds to PDK4 in podocytes from mouse and inhibits the expression of PDK4 mRNA and protein. Inhibition of PDK4 can reduce the activation of VEGFA and downregulate the inflammation and cell apoptosis (Table 1). The results of several studies showed that stem cell products inhibit VEGFA expression through multiple pathways, thereby attenuating HG-induced damage to podocytes.

Li et al. (31) reported that AD-MSC-CM reduced podocyte apoptosis induced by HG, downregulated activated caspase-3, increased epithelial growth factor (EGF), and prevented the rearrangement and downregulation of synaptopodin. However, it did not affect the levels of glial cell line-derived neurotrophic growth factor (GDNF), insulin-like growth factor binding protein and placental growth factor (Table 1). However, a study pointed out that GDNF promotes mouse podocyte survival *in vitro* and protects mouse podocytes from apoptosis (49). Zhang et al. (14) confirmed this and found that HG can reduce podocytic synaptopodin, and AD-MSC-CM can increase synaptopodin expression in podocytes. After blocking GDNF in AD-MSC-CM with GDNF-NtAb, the therapeutic effect of podocytic synaptopodin was partly abolished (Table 1). These studies demonstrated that stem cell products reduce podocytic apoptosis by modulating cytokines.

Autophagy is a lysosomal degradation pathway in cells for maintaining cellular homeostasis and cellular health under various stress conditions (50). Evidence suggests that podocytes have high levels of basal autophagy, which may be a mechanism to maintain cellular homeostasis (51). Jin et al. (32) showed that AD-MSC-EVs can inhibit p-mTOR/mTOR, Smad1, p62, and apoptosis, but they can increase Beclin1 and LC3 (Table 1). AD-MSC-EVs ameliorate podocyte damage by inhibiting the miR-486/Smad1/mTOR signaling pathway. This suggested that stem cell products can regulate the autophagy of podocytes.

HG may induce podocyte epithelial-mesenchymal transition (EMT) through multiple pathways (52). Jin et al. (33) showed that, with podocyte dysfunction, several EMT-related miRNAs, including miR-3066-5p, miR-879-5p, miR-251-5p, and miR-7a-5p, were increased by the addition of AD-MSC-EVs (Table 1). Potentially, AD-MSC-EVs can mediate the shuttling of miR-215-5p to podocytes, possibly through inhibiting the transcription of zinc finger E-box-binding homeobox 2 (ZEB2), thereby attenuating EMT of podocytes.

Stem cell products ameliorate fibrosis in mesangial cells

The signaling pathway of TGF-β can play an important role in the fibrogenesis, especially in DKD renal fibrosis, and can be activated by high glucose (53). Endothelin-1 (ET-1) promotes fibrosis and inflammation in DKD (54). ET-1 and TGF-β1 induce collagen I production by fibroblasts (55). Li et al. (34) found that blocking TGF-β1 by UC-CM inhibited the expression of collagen I and fibronectin in mesangial cells treated with HG. Antifibrotic effects of MSC paracrine in DN may be detected by EVs shed by MSCs. BM-MSC-CM treatment remarkably reduced expressions of TGF-β and TGF-β-induced glucose transporter 1, thus inhibiting fibrosis and oxidative stress. A large amount of hepatocyte growth factor (HGF) was detected in CM, and the effects of CM on TGF-β and TGF-β-induced glucose transporter expression could be blocked by the addition of neutralizing antibodies

TABLE 1 Characteristics of assessing the efficacy of MSC products for DKD *in vitro*.

Author	Cell model	Stem cell type	Treatment effect
	Podocyte		
Jiang et al. (27)	HPDCs	USC-EVs	Reduce podocyte apoptosis (BMP-7, VEGF, TGF-β and angiogenin↑)
Duan et al. (28)	HPDCs	HUC-EVs	Increase podocyte viability and reduce rate of apoptosis (miR-16-5p↑→ VEGFA↓)
Duan et al. (29)	MPC5	ASC-EVs	Inhibit podocyte apoptosis (miR-26a-5p↑→ TLR4↓→ NF-κB/VEGFA↓)
Zhao et al. (30)	MPC5	ASC-CM	Inhibit podocyte apoptosis and inflammation (miR-15b-5p↑→ PDK4↓→ VEGFA↓)
Li et al. (31)	MPC5	ASC-CM	Reduce podocyte apoptosis (EGF↑)
Zhang et al. (14)	MPC5	ASC-CM	Reduce podocyte apoptosis (GDNF↑)
Jin et al. (32)	MPC5	ASC-EVs	Promote autophagy and inhibit podocyte apoptosis (miR-486↑→ Smad1↓→ mTOR↓)
Jin et al. (33)	MPC5	ASC-EVs	Attenuate EMT of Podocytes (miR-215-5p↑→ ZEB2↓)
	Mesangial cells		
Li et al. (34)	SV40-MES-13	USC-CM	Alleviate Fibrosis (MAPK↓ and PI3K/Akt↓→ MMP2 and MMP9↑)
Lv et al. (35)	HBZY-1	BMSC-CM	Inhibit fibrosis and oxidative stress and reduce the expression of GLUT1
Bai et al. (36)	HBZY-1	BMSC-CM	Inhibit fibrosis and inflammation (LXA4↑→ TGF-β↓)
Gallo et al. (37)	MCs	BMSC-EVs HLSC-EVs	Inhibit fibrosis and interference with mitochondrial dysfunction (miR-222↑/miR-21↓) →, STAT5A↓→, TGF-β↓)
Hao et al. (38)	GMC	ASC-EVs	Inhibit fibrosis and reduce apoptosis (miR-125a↑→ HDAC1/ET1↓)
	Renal tubular epithelial cells		
Nagaishi et al. (39)	PTECs	BMSC-CM	Anti-apoptotic and anti-degenerative (TGF-β1↓, lectin and ZO-1↑)
Park et al. (40)	NRK-52E	USC-CM	Inhibited ECM and EMT (TGF-β1↓)
Zhong et al. (41)	HK-2	HUC-MVs	Reverse EMT by restarting the blocked cell cycle (miR-451a↑→ P15 and P19↓)
Rao et al. (42)	HK-2	SHED-CM BMSC-CM	Inhibit AGE-induced EMT (E-cadherin↑, fibronectin and vimentin↓)
Ali et al. (43)	HK-2	WJMSCs-CM	Attenuate oxidative stress-mediated apoptosis and fibrosis (CHIP↑→ MAPK↓)
Lee et al. (44)	HK-2	USC-CM	Reverse mitochondrial dysfunction (Arg↓→ M1 macrophages↓→ NO, IL-6, TNF- IL-1↓)
Konari et al. (45)	NRK-52E	BMSC-CM	Inhibit apoptosis and reduce ROS production by transferring mitochondria

ASC, adipose-derived MSCs; BMSC, bone marrow-derived MSCs; GMC, rat glomerular mesangial cells; HBZY-1, The rat glomerular mesangial cell line; HK-2, human proximal tubular epithelial; HLSC, human liver stem-like cells; HPDCs, Human podocytes; HUC, human urine-derived MSCs; MCs, Human mesangial cells; MPC5, A mouse podocyte clone 5; NRK-52E, renal tubularduct epithlialcells of rat; PTECs, proximal tubular epithelial cells; SHED, Stem cells from human exfoliated deciduous teeth; SV40-MES-13, mouse mesangial cell; WJMSCs, Wharton's jelly-derived MSCs; USC, umbilical cord-derived MSCs; AGEs, advanced glycation end products; Arg1, arginase-1; BMP-7, bone morphogenetic protein-7; CHIP, Carboxyl terminus of HSP70 interacting protein; CM, conditioned medium; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; ET-1, endothelin-1; GDNF, glial cell-derived neurotrophic growth factor; HDAC1, histone deacetylase 1; HGF, hepatocyte growth factor; LXA4, lipoxin A4; MAPK, mitogen-activated protein kinase; MMP, metalloproteinase; PDK4, Pyruvate dehydrogenase kinase 4; ROS, reactive oxygen species; STAT1, Signal Transducers and Activators of Transcription-1; TLR4, toll-like receptor 4; VEGF, vascular endothelial growth factor; ZEB2,Zinc finger E-box-binding homeobox 2; ZO-1, zona occludens protein-1.

against HGF (Table 1) (35). This indicated that HGF in CM alleviates mesangial cells' fibrosis and oxidative stress. Co-culture of BM-MSCs and mesangial cells alleviated cell fibrosis by targeting lipoxin A4 (LXA4) to modulate TGF-β/Smad signaling (36). STAT5A was identified as a transcriptional regulator of miR-21, which in turn affects collagen production and TGF-β expression in mesangial cells. MSC-EV-miR-222 regulates STAT5 expression and indirectly regulates TGF-β expression (37). Hao et al. (38) found that AD-MSC-EVs inhibit the histone deacetylase 1 (HDAC1)/ET1 axis by secreting miR-125a and suppressing IL6, collagen I and fibronectin levels in HG-treated mesangial cells. All these results suggested that MSC-EVs alleviate mesangial cell fibrosis by regulating the expression of TGF-β and ET-1 (Table 1).

Stem cell products ameliorate fibrosis in renal tubular epithelial cells

Renal tubulointerstitial fibrosis, characterized by EMT of renal tubular epithelial cells (RTEC), is a major cause of diabetic renal fibrosis (56). Important roles of different tubular responses, such as partial EMT, cell cycle arrest and metabolic defects are involved in renal fibrosis (57). Nagaishi et al. (39) found that MSC-EVs can reduce intracellular adhesion molecule-1 (ICAM-1) and TGF-β1 in RTEC isolated from streptozotocin (STZ)-induced diabetic rats, and zona occludens protein-1 (ZO-1) expression was increased in RTEC cultured with EVs or MSCs. UC-MSC-CM inhibited TGF-β1-induced EMT and

extracellular matrix accumulation (ECM) in RTEC (NRK-52E) (Table 1) (40). Zhong et al. (41) further found that MSC-EV-miR-451a reverses EMT by inhibiting P15 and P19 to restart the blocked cell cycle (Table 1). Inhibition of fibrosis by regulating the cell cycle by miRNA may be a new therapeutic modality.

Advanced glycation end products (AGEs) induce apoptosis and increase the expression of inflammatory and fibrotic genes in renal tubular cells (58). Hyperglycemia-induced reactive oxygen species (ROS) generation activates mitogen-activated protein kinases (MAPKs), which are involved in the EMT of RTEC (HK2) (59, 60). Co-cultured stem cells from human exfoliated deciduous teeth inhibit AGE-induced EMT in HK-2 cells (Table 1) (42). Ali et al. (43) reported that the protein expression of Carboxyl terminus of HSP70 interacting protein (CHIP) was reduced under HG treatment, which can limit the therapeutic potential and survivability of Wharton's jelly-derived MSCs (WJMSCs). CHIP-overexpressing WJMSCs attenuate fibrosis and cell apoptosis mediated by hyperglycemia-induced oxidative stress in HK-2 cells *via* activation of the MAPKs (Table 1). This proves that MSC-EVs can inhibit the production of AGEs and ROS and downregulate the fibrosis of renal tubular epithelial cells.

Mitochondria have been recognized as key regulators of inflammation, cell death, metabolism and ROS production (61). M1 macrophages express pro-inflammatory cytokines, and M2 macrophages are thought to regulate inflammatory responses and promote tissue repair (62). Arginase-1 (Arg1) is a marker of M2 macrophages, and co-culture of macrophages with MSCs increases Arg1 and decreases the expression of M1 markers. UC-MSCs reverse mitochondrial function in HK-2 cells by inducing Arg1 in macrophages (44). It should be noted that AD-MSCs cannot reverse mitochondrial dysfunction, which may indicate that the beneficial effect of MSCs is limited to umbilical cord blood. Konari et al. (45) showed that BM-MSCs also transfer their mitochondria to damaged RTEC when co-cultured *in vitro*, inhibiting apoptosis and reducing ROS production of damaged RTEC (Table 1).

MSC-EVs protect HG-induced damaged kidney cells through multiple pathways, suggesting that MSC-EVs may be a potential therapeutic modality for DKD (Figure 2).

Efficacy of MSC-EVs for DKD: A preclinical model

Intravenous injection of MSC-EVs can improve renal function and histological damage in diabetic animals. Hyperglycemia is a major factor in the occurrence of DKD. EVs secreted from AD-MSCs, BM-MSCs and UC-MSCs are able to reduce blood glucose. Furthermore, EVs can reduce serum creatinine (SCr), blood urea nitrogen (BUN), urinary protein (URPO), and urine albumin-to-creatinine ratio (UACR), and increase creatinine clearance (CCR) (28, 38, 63). Hyperlipidemia is an important risk factor for vascular complications of chronic kidney disease (64). BM-MSC-EVs reduced total cholesterol (TC) and triglycerides (TG) in a DKD mouse model (65, 66). Jiang et al. (27) found that HU-MSC-EVs could reduce the urinary microalbumin excretion and urine volume of DKD rats.

Duan et al. (29) showed that AD-MSC-EVs notably alleviated the histopathological changes associated with DKD, such as reducing ECM accumulation in their kidney tissues and the thickening of basement membrane. Jiang et al. (27)found that UC-MSC-EVs could prevent cell apoptosis in diabetic rats. Further, UC-MSC-EVs treatment significantly ameliorated mesangial expansion and promoted endothelial cell proliferation from glomerulus in the early stages of impairment in DKD kidney. Ebrahim et al. (63) found that injection of EVs in DKD rats reduced histological damage. EVs alleviated diffuse thickening of glomerular basement membrane and extensive fusion and disappearance of foot process.

Inflammation and fibrosis play a key role in the pathogenesis of DKD. Xiang et al. (67) found that UC-MSC-CM or UC-MSC-EVs suppressed IL-1β, TNF-α, IL-6, and TGF-β in HG-injured human renal glomerular endothelial cell line (hrGECs) and RTECs (HK2 and NRK-52E). Duan et al. (28) reported that HU-MSC-EVs can reduce the expression of VEGFA, monocyte chemoattractant protein-1 (MCP-1), TNF-α and TGF-β1 in diabetic mice. This means that the UC-MSC product can relieve inflammation in diabetic mice. MCP-1 is also known as the C-C motif chemokine ligand 2 (CCL2). CCL2 can stimulate the production of TGF-β1 in mesangial cells and macrophages, and TGF-β1 feeds back to increase the CCL2 expression in mesangial cells (68). Substantial evidence indicates that TGF-β/Smad signaling takes part in the development and progression of fibrosis of kidney (69). Hao et al. (38)found that AD-MSC-EVs reduced the protein expression of the Col-I (fibrosis-related marker) and suppressed mesangial hyperplasia in DKD rats (Table 2). Mao et al. (65) found that BM-MSC-EVs miR-let-7a inhibited the expression of N-cadherin and vimentin (Table 2). Furthermore, Nagaishi et al. (39) showed that BM-MSC-CM can reduce TNF-α, ICAM-1 and increase ZO-1 (Table 2). Zhong et al. (41) showed that UC-MSC-EVs reduced α-SMA, increased E-cadherin and prevented histological damages (Table 2). Grange et al. (70) reported that human liver stem-like cells (HLSC)-EVs and BM-MSC-EVs down-regulate genes involved in the pathogenesis of fibrosis, such as tissue inhibitor of metalloproteinases (TIMP), metalloproteinase 3 (MMP3), collagen I, TGF-β and α-SMA, the FAS ligand, CCL3, and Snail (Table 2). This indicates that injection of MSC-EVs from various sources can alleviate fibrosis in diabetic animals.

In addition to being anti-inflammatory and anti-fibrotic, MSC-EVs protect DKD animals by other means. With the effecting by increased oxidative stress and reduced NO bioavailability, endothelial dysfunction is a hallmark feature of type 2 diabetes mellitus and DKD (71). Mao et al. (65) found that

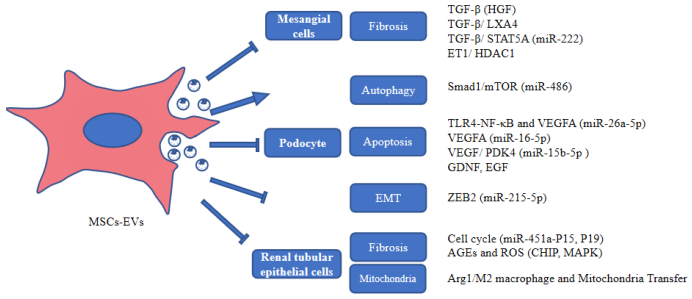

FIGURE 2
The role of MSC-EVs in protecting kidney cells. AGEs, advanced glycation end products; Arg1, arginase-1; EGF, epidermal growth factor; GDNF, glial cell-derived neurotrophic growth factor; HDAC1, histone deacetylase 1; HGF, hepatocyte growth factor; LXA4, lipoxin A4; MAPK, mitogen-activated protein kinase; PDK4, Pyruvate dehydrogenase kinase 4; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; ZEB2, Zinc finger E-box-binding homeobox 2.

BM-MSC-EVs suppress oxidative stress, based on decreases in NO and MDA content, and elevations of GSH-Px and SOD activities. EVs injection can alleviate the oxidative stress reaction in diabetic mice and play a protective role in DKD through down-regulation of USP22. Autophagy, a conserved and important "self-eating" pathway, is an important mechanism for maintaining glomerular and tubular homeostasis, and is involved in various aspects of renal injury, aging and disease (72). Ebrahim et al. (63) reported that BM-MSC-EVs can inhibit mTOR, and S6K1 protein expression, and increase Beclin-1, LC3-I, LC3-II, and p62 protein expression. MSC-EVs ameliorate DKD by modulating the mTOR-related autophagy pathway (Table 2). Duan et al. (29) reported that Bcl-2 protein was significantly increased by AD-MSC-EVs, whereas the protein expressions of cleaved caspase-3, caspase-3, and Bax were reduced. MiR-26a-5p produced by AD-MSC-EVs not only reduced podocyte apoptosis *in vitro*, but also reduced apoptosis in spontaneously diabetic mice.

Overall, through multiple studies injected with stem cell products from different sources, we found that stem cell products can alleviate kidney damage in animal models of DKD (Table 1).

TABLE 2 Characteristics of preclinical studies assessing the efficacy of MSC products for DKD *in vivo*.

Author	Stem cell type	Model	Treatment		Treatment effect
Hao et al. (38)	ASC-EVs	STZ SD	Exos (50μg) *via* tail vein injection	Twice a week for 3 weeks	miR-125a protected DKD in rats through inhibiting the HDAC1/ET1 axis
Mao et al. (65)	BMSC-EVs	STZ SD	Exos (100μg) *via* tail vein injection	Once a week for 12 weeks	miR-let-7a inhibited apoptosis and oxidative stress in renal cells and suppressed the expression of N-cadherin and vimentin
Nagaishi et al. (39)	BMSC-CM	STZ mice	CM (2mg/kg) *via* tail vein injection	Once a day for 8 weeks	Anti-inflammation and inhibition of EMT (TNF-α, p38-MAPK, ICAM-1, TGF-β↓ and ZO-1↑)
Zhong et al. (41)	HUC-EVs	STZ mice	MVs (1.5mg/kg) *via* tail vein injection	Once a week for 8 weeks	miR-451a reduced renal fibrosis through down-regulation of the P15INK4b and P19INK4d
Grange et al. (70)	HLSC-EVs BMSC-EVs	STZ mice	EVs(1×10^{10} particles) *via* tail vein injection	Once a week for 4 weeks	EVs down regulated genes involved in the development of fibrosis (MMP3, collagen I, TIMP, SNAI1, CCL3, Serpina1, interferon γ, Fas Ligand↓)·
Duan et al. (29)	ASC-EVs	C57BL/KsJ db/db	–	Once a week for 12 weeks	miR-26a-5p reduced the pathological symptoms and cell apoptosis
Jiang et al. (27)	USC-EVs	STZ SD	Exos (100μg) *via* tail vein injection	Once a week for 12 weeks	Anti-apoptosis, promoted glomerular endothelial cell proliferation and ameliorated mesangial expansion
Ebrahim et al. (63)	BMSC-EVs	STZ SD	Exos (100μg/kg) *via* tail vein injection	Once a day for 4 weeks	Induction of autophagy through the mTOR signaling pathway

ASC, adipose-derived MSCs; BMSC, bone marrow-derived MSCs; HLSC, human liver stem-like cells; HUC,human urine-derived MSCs; USC, umbilical cord-derived MSCs; CCL3, C-C motif chemokine ligand 3; CM, conditioned medium; EMT, epithelial-mesenchymal transition; ET-1, endothelin-1; EVs, extracellular vesicles; HDAC1, histone deacetylase 1; ICAM-1, intracellular adhesion molecule-1; MAPK, mitogen-activated protein kinase; MVs, microvesicles; MMP3, metalloproteinase 3; TIMP, tissue inhibitor of metalloproteinases; SD, Sprague-Dawley rat; STZ, streptozotocin; ZO-1, zona occludens protein-1.

Effect of stem cell extracellular vesicles on inflammatory factors and their potential signaling pathways

Phosphorylation of signal transducers and activators of transcription-1 (STAT1) is induced by multiple TLRs (TLR2, TLR4, TLR9). Tumor necrosis factor receptor-associated factor-6 (TRAF6) is a most important factor to activate TLR signaling. After activation of TRAF6, STAT1 is phosphorylated and translocated to the nucleus (73). Zhang et al. (74) found that UC-MSCs-derived miR-146a-5p can target the TRAF6 and significantly decreased the expression of p-STAT1. They identified that miR-146a-5p targeted the signaling pathway of TRAF6-STAT1 to inhibit renal inflammation and restore the function of kidney by promoting the polarization of M2 macrophage. Transcription factors, such as NF-κB, can regulate inflammation, immunological responses and cell proliferation (75). In DKD, the transposition of NF-κB into the nucleus can activate its target genes, including the inflammatory mediators of its downstream, such as nitric oxide synthase, TNF-β1, IL-1 and ICAM-1, which subsequently cause persistent and increased inflammation leading to overexpression of fibronectin and ECM accumulation in mesangial cells (76). Duan et al. (29) reported that AD-MSC-EV-miR-26a-5p alleviated both the ECM accumulation in kidney tissues and the thickening of basement membrane, and inhibited apoptosis in mouse podocytes *in vitro* by inhibiting signaling pathways of NF-κB/VEGFA and TLR4.

Autophagy promotes the degradation of excess or malfunctioning cellular components, including invasive microorganisms, misfolded proteins, damaged organelles and cells themselves (77). In DKD, autophagy represents the cooperation of related gene products of multiple autophagy (23). In general, mTORC1 is regarded as a negative regulator for the autophagy. Enhanced mTORC1 activity was observed in type 1 and type 2 DKD diseases from animal models. Treatment with rapamycin (an inhibitor of mTORC1) can suppress the progression of DKD disease induced by STZ in type 1 and type 2 diabetes from rats or mice (78, 79). Injection of MSC-EVs into the tail vein of DKD rats resulted in significant up-regulation of autophagy-related proteins (LC3II and Beclin-1), and significant down-regulation of mTOR gene expression. The results indicated that BM-MSC-EVs enhance autophagy by inhibiting mTOR (63). Jin et al. (32) found that AD-MSC-EVs inhibited p62/LC3 and mTOR signaling pathways, increased the levels of autophagy-related proteins, and ameliorated cell damage of podocyte by inhibiting the signaling pathway of miR-486/Smad1/mTOR. Cai et al. (80) showed that miR-125b from MSC-EVs could induce the cell autophagy to inhibit HKCs apoptosis induced by HG *via* signaling pathway of Akt. Collectively, multiple miRNAs in EVs inhibited mTORC1 expression by inhibiting AKT and finally upregulated autophagy.

It is well known that the signaling pathway of TGF-β can play an important role in fibrogenesis and also promotes fibrosis in DKD (81). Additionally, the signaling pathway of TGF-β/Smad also affects DKD through cross-talk with other pathways, such as the MAPK and PI3K/Akt signaling pathways (82). The TGF-β/Smad signaling pathway is notably activated in renal fibrosis. Smad3 exhibits a marked collagen deposition and contributes to the progression of renal fibrosis in db/db mice, and Smad3 knockout inhibits this process (83). Nagaishi et al. (39) reported that MSC-CM inhibits tubulointerstitial fibrosis in a model of DKD by reversing the endogenously elevated or ectopically expressed TGF-β. Furthermore, Bai et al. (36) demonstrated that MSC-CM reversed DKD *via* LXA4 by targeting the TGF-β/Smad pathway and pro-inflammatory cytokines. Liu et al. (84) found that MSCs modified with angiotensin-converting enzyme 2 (ACE2) to target the damaged kidney and enhance the expression of ACE2. The modified MSCs secreted soluble ACE2 protein into the culture medium. The upregulated ACE2 can degrade the Ang II into the Ang1-7, and MSCs-ACE2 is more beneficial when compared with MSCs alone in downregulating Ang II and upregulating Ang1-7. MSCs-ACE2 inhibited the deleterious effects of the accumulation of Ang II and inhibited the TGF-β/Smad pathway. Li et al. (34) found that MSC-CM alleviated renal fibrosis in a DKD model by blocking myofibroblast transdifferentiation mediated by the signaling pathway of TGF-β1/Smad2/3. MSC-CM also inhibited the proliferation of mesangial cell mediated by signaling pathways of PI3K/Akt and MAPK, and enhanced the expression of MMPs. Multiple investigations have indicated that MSC-EVs improve renal fibrosis which mediated TGF-β pathway and PI3K/Akt and MAPK signaling pathway by inhibiting the expression of TGF-β. (Figure 3).

Conclusion

The importance of DKD has been increasingly acknowledged and treatment methods have been updated and improved. However, in the past 20 years, the incidence and prognosis of DKD have not been effectively controlled. MSC-based therapy brings prospective treatment to DKD. Various growth factors and miRNAs contained in MSC-EVs seem to play an important role in the therapeutic effect of MSCs. Some studies have pointed out that MSC-EVs not only protect podocytes, renal tubular epithelial cells and mesangial cells from HG-induced injury, but also protect against DKD in animals. Moreover, some studies have shown that the treatment efficacy of ACE2-modified MSCs is more effective than MSCs alone, and MSCs combined with miRNAs treatment can enhance the protective effect of MSCs. At the same time, MSC-EVs also alleviated the DKD in animals through various regulatory signaling pathways (NF-κB, TLR, mTOR, MAPK, PI3K/Akt, TGF-β/Smad). However, it is difficult to find a

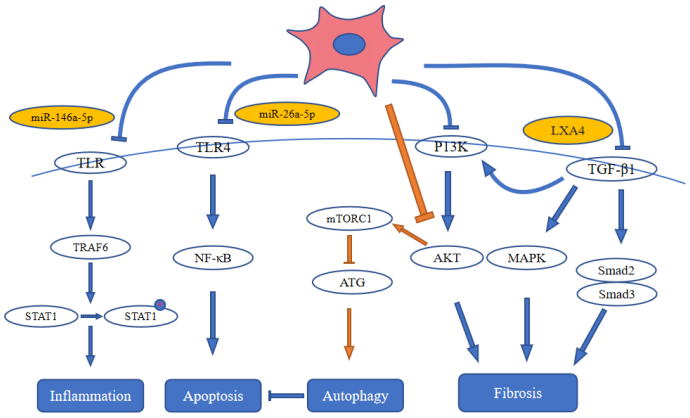

FIGURE 3
Stem cell therapy influences signal pathways in diabetic kidney disease. ATG: autophagy-related gene; LXA4: lipoxin A4; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor kappa-B; STAT1: Signal Transducers and Activators of Transcription-1; TLR4: toll-like receptor 4; TRAF6: tumor necrosis factor receptor-associated factor-6.

unified standard for the treatment of DKD with MSC extracellular vesicles. Different stem cell-derived extracellular vesicles, injection doses and frequencies are used in different experiments. Therefore, more research is needed to optimize the different MSC products, injection frequencies and doses to treat DKD.

Author contributions

YDL and TZ looked up a lot of pertinent papers and wrote the manuscript. QY, JW, XC and YPL reviewed and checked the article. TZ modified and polished the article, and reviewed the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

ACE2	angiotensin-converting enzyme 2
AD-MSCs	adipose-derived MSCs
AGEs	advanced glycation end products
Arg1	arginase-1
BM-MSCs	bone marrow-derived MSCs
BMP-7	bone morphogenetic protein-7
CCL2	C-C motif chemokine ligand 2
CHIP	Carboxyl terminus of HSP70 interacting protein
DKD	diabetic kidney disease
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ESRD	end-stage chronic kidney disease
ET-1	endothelin-1
EVs	extracellular vesicles
Exos	exosomes
GDNF	glial cell-derived neurotrophic growth factor
HDAC1	histone deacetylase 1
HG	high glucose
HGF	hepatocyte growth factor
HLSC	human liver stem-like cells
HRGECs	human renal glomerular endothelial cell lines
HU-MSCs	human urinederived MSCs
ICAM-1	intracellular adhesion molecule-1
LXA4	lipoxin A4
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MMP3	metalloproteinase 3
MSCs	mesenchymal stromal cells
MVs	microvesicles
MSC-Exos	MSC exosomes
NF-kB	nuclear factor kappa-B
PDK4	Pyruvate dehydrogenase kinase 4
PTECs	proximal tubular epithelial cells
RTEC	renal tubular epithelial cells
ROS	reactive oxygen species
STAT1	Signal Transducers and Activators of

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The Chronic Kidney Disease Epidemiology Collaboration equations perform less well in an older population with type 2 diabetes than their non-diabetic counterparts

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Objectives: The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations are based on creatinine alone (CKD-EPIcr), cystatin C alone (CKD-EPIcys) and combined creatinine and cystatin C (CKD-EPIcr-cys). It remains unclear whether these equations perform differently in older adults with type 2 diabetes than they do in non-diabetic older individuals.

Methods: This single-center cross-sectional study was performed in adults aged ≥ 65 years between January 2019 and December 2021. Glomerular filtration rate (GFR) was measured by technetium-99m-diethylene triamine pentaacetic acid (^{99m}Tc-DTPA) renal dynamic imaging. The bias (difference between measured and estimated GFR), precision [interquartile range (IQR) of the median difference between measured GFR and estimated GFR] and accuracy P30 (percentage of estimated GFR within 30% of measured GFR) were considered the criteria of equation performance.

Results: Finally, 476 participants were enrolled, including 243 adults with type 2 diabetes and 233 non-diabetic adults. The mean age of the included participants was 71.69 ± 6.4 years and 262 (55%) were male. The mean measured GFR was 49.02 ± 22.45 ml/min/1.73 m^2. The CKD-EPIcr-cys equation showed significantly greater bias and lower accuracy (P30) in individuals with diabetes than in the non-diabetic group (median bias, 4.08 vs. 0.41 ml/min/1.73 m^2, respectively, $p < 0.05$; P30, 63.78% vs. 78.54%, respectively, $p < 0.05$). The precision IQR indicated that CKD-EPIcr-cys had also lower precision in individuals with diabetes than in the non-diabetic controls (17.27 vs. 15.49 ml/min/1.73 m^2, respectively). Similar results were observed for CKD-EPIcr and CKD-EPIcys equations. The P30 of all three equations failed to reach 80% in diabetic and non-diabetic groups.

Conclusions: The performance of the CKD-EPI equations was lower in a group of patients aged ≥ 65 years with type 2 diabetes than in non-diabetic

counterparts. However, each equation still had limitations regarding accuracy in older adults with or without diabetes.

KEYWORDS

CKD-EPI equations, glomerular filtration rate, type 2 diabetes, elderly, creatinine, cystatin C

Introduction

The number of people older than 65 years with diabetes worldwide was 135.6 million in 2019, and is projected to increase to 276.2 million by 2045 (1). The prevalence of diabetes in adults has more than tripled over the last two decades, from an estimated 151 million (4.6% of the global population) in 2000 to 537 million (10.5%) today (2, 3). The aging of the population and the increase in the prevalence of diabetes are two important factors associated with the increased incidence of chronic kidney disease (CKD) (4). Precise estimation of glomerular filtration rate (GFR) is critical for diagnosis, classification, and management of patients with CKD, particularly in those with comorbid diabetes (5).

The GFR is regarded as the best overall index of kidney function in both health and disease. However, measurement of GFR using clearance of inulin (6), iohexol (7), or ^{125}I-iothalamate (8) is invasive and may be too inconvenient and costly for use in everyday practice. Technetium-99m-diethylene triamine pentaacetic acid (^{99m}Tc-DTPA) renal dynamic imaging (9), which is recommended for GFR measurement by the Nephrology Committee of the Society of Nuclear Medicine (10), has been widely used in clinical practice. However, as ^{99m}Tc-DTPA is also inconvenient, the use of GFR-estimating equations has become more common.

In 2009, the CKD Epidemiology Collaboration (CKD-EPI) initially developed a GFR-estimating equation based on serum creatinine for use in individuals with and without kidney function loss (11). Given the association between aging and physiological changes in the kidneys as well as the potential effects of muscle mass on serum creatinine, the CKD-EPI then developed two other equations based on cystatin C alone and in combination with creatinine in 2012, and demonstrated that the combined creatinine-cystatin C equation had better performance than equations based on either of these markers alone (12). However, the applicability of these equations in older Chinese adults with diabetes is unknown. This study was conducted to assess the performance of three CKD-EPI equations in a population of older individuals with type 2 diabetes in comparison with their non-diabetic counterparts.

Patients and methods

Study design

This case-control study was conducted at China-Japan Friendship Hospital, Beijing, China, between January 2019 and December 2021. Participants, who were diagnosed with acute kidney failure, receiving dialysis, or with dehydration or fluid overload were excluded. Type 2 diabetes was diagnosed according to the 2022 American Diabetes Association (ADA) criteria (13).

Data collection and measurements

Clinical information, including laboratory (serum levels of creatinine, cystatin C and albumin) and demographic data (age, sex, and disease history) were obtained from the Electronic Medical Record System of our center. Serum creatinine level was determined by enzymatic kinetic assay under fasting conditions, and cystatin C was measured using a latex particle-enhanced turbidimetric immunoassay. Patients' heights and weights were also recorded.

The reference GFR was measured using ^{99m}Tc-DTPA renal dynamic imaging. The results were normalized to a body surface area (BSA) of 1.73 m^2, as described by the Dubois method: BSA (m^2) = 0.007184 × body weight (kg)$^{0.425}$ × height (cm)$^{0.725}$ (14).

CKD-EPI equations

The eGFR was calculated using the Creatinine Equation (CKD-EPIcr 2009) (11), Cystatin C Equation (CKD-EPIcys 2012), and Creatinine-Cystatin C Equation (CKD-EPIcr-cys 2012) (12). The CKD-EPIcr equation (2009) is expressed as follows: $141 \times \min(Scr/\kappa, 1)^{\alpha} \times \max(Scr/\kappa, 1)^{-1.209} \times 0.993^{age}$[× 1.018 if female] [× 1.159 if Black], where Scr is serum creatinine, κ is 0.7 for females and 0.9 for males, α is −0.329 for females and −0.411 for males, min is the minimum of Scr/κ or 1, and max is the maximum of Scr/κ or 1 (11). The CKD-EPIcys equation (2012) is expressed as follows: $133 \times \min(Scys/0.8, 1)^{-0.499} \times \max(Scys/0.8, 1)^{-1.328} \times 0.996^{age}$ [× 0.932 if female], where Scys is serum cystatin C, min indicates

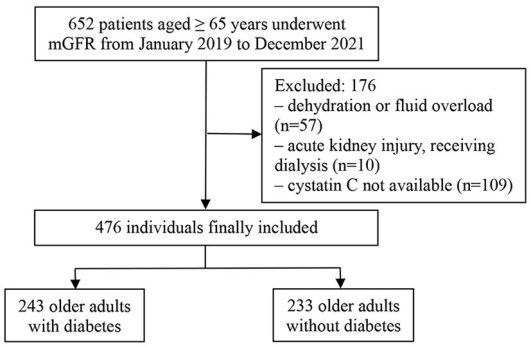

FIGURE 1
Flowchart of the study patients. mGFR, measured glomerular filtration rate; PSM, propensity score matching.

the minimum of Scys/κ or 1, and max indicates the maximum of Scys/κ or 1 (12). The CKD-EPIcr-cys equation (2012) is expressed as follows: $135 \times \min(\text{Scr}/\kappa, 1)^{\alpha} \times \max(\text{Scr}/\kappa, 1)^{-0.601} \times \min(\text{Scys}/0.8, 1)^{-0.375} \times \max(\text{Scys}/0.8, 1)^{-0.711} \times 0.995^{\text{age}}$ [× 0.969 if female] [× 1.08 if Black], where Scr is serum creatinine, Scys is serum cystatin C, κ is 0.7 for females and 0.9 for males, α is −0.248 for females and −0.207 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1 (12).

Statistical analysis

Three criteria were considered in the evaluation of equation performance: bias, precision, and accuracy. Bias was expressed as the median difference (MD) between measured GFR (mGFR) and eGFR, where a negative or positive bias indicated overestimation or underestimation of eGFR, respectively. Precision was expressed as the interquartile range (IQR) of the difference between mGFR and eGFR. Accuracy was considered under two criteria: root mean square error (RMSE), defined as the square root of the average squared difference of eGFR and mGFR on a logarithmic scale; and P30, defined as the percentage of estimates within 30% of mGFR. When P30 is > 90%, the equation fulfills the requirements of clinical interpretation (6, 15, 16). By using the Wilcoxon, McNemar, and χ^2 tests, differences between equations were compared. Bland-Altman analysis was performed to examine the agreement between mGFR and eGFR. The smaller the width between 95% limits of agreement (LOA), the better agreement. Statistical analyses were conducted using SPSS (version 23.0; IBM Corp., Armonk, NY, USA), and MedCalc (version 20.0.15; MedCalc, Mariekerke, Belgium). Statistical significance was defined as a value of $p < 0.05$.

Results

Characteristics of the study population

Of an initial 652 older adults, 476 fulfilled the study criteria (Figure 1), 262 (55%) of whom were male. The mean age of the participants was 71.69 ± 6.4 years. The mean mGFR was 49.02 ± 22.45 ml/min/1.73 m^2. Participants were divided according to the presence or absence of type 2 diabetes into the diabetic group (243 participants) and non-diabetic group (233 participants). Table 1 shows the demographic and main laboratory data of the participants. Older adults with diabetes had significantly lower mGFR than the non-diabetic group (46.17 ± 23.3 vs. 51.99 ± 22.28 ml/min/1.73 m^2, respectively, $p = 0.005$). In addition, diabetic participants had a slightly higher level of body mass index than those without diabetes (25.08 ± 3.03 vs. 24.35 ± 3.34 kg/m^2, respectively, $p = 0.027$). However, there were no significant differences in age, sex, and serum albumin between the two groups (Table 1).

Performance of equations in individuals with or without diabetes

Table 2 shows the performance of the three equations in individuals with and without type 2 diabetes, determined by calculating the bias, precision and accuracy. In the overall population, the bias of CKD-EPIcr was −0.81 ml/min/1.73 m^2, which was smaller than each of CKD-EPIcys (3.91 ml/min/1.73 m^2) and CKD-EPIcr-cys (2.24 ml/min/1.73 m^2). Regarding accuracy P30, only CKD-EPIcr-cys exceeded 70%, which was significantly higher than either CKD-EPIcr (66.81%) or CKD-EPIcys (64.91%). In other words, CKD-EPIcr had the smallest bias in the overall population, but CKD-EPIcr-cys achieved the better precision and accuracy.

In individuals with diabetes, the median bias between CKD-EPIcr (1.26 ml/min/1.73 m^2) and each of CKD-EPIcys (5.51 ml/min/1.73 m^2) and CKD-EPIcr-cys (4.08 ml/min/1.73 m^2) was significant (less bias in the former); there was also significant difference in median bias between CKD-EPIcys and CKD-EPIcr-cys (4.08 ml/min/1.73 m^2). Precision IQR (P75–P25) demonstrated that the CKD-EPIcr-cys equation had higher precision (17.27 ml/min/1.73 m^2) than CKD-EPIcr and CKD-EPIcys equations (18.88 and 18.51 ml/min/1.73 m^2, respectively). The differences in accuracy (P30) between the three CKD-EPI equations were not statistically significant (62.55, 60.08, and 63.78%, respectively). CKD-EPIcr, CKD-EPIcys, and CKD-EPIcr-cys had similar RMSE values (0.199, 0.194, and 0.193, respectively). The aforementioned results indicate that the three equations had the similar precision and accuracy although CKD-EPIcr had the smallest median bias.

TABLE 1 Demographic and clinical data for participants aged 65 years and older*.

	Overall ($n = 476$)	Individuals with diabetes ($n = 243$)	Individuals without diabetes ($n = 233$)	p
age, years	71.69 ± 6.40	71.54 ± 6.31	71.84 ± 6.51	0.60
males, *n* (%)	262 (55.0)	130 (53.5)	132 (56.7)	0.49
BMI, kg/m^2	24.72 ± 3.20	25.08 ± 3.03	24.35 ± 3.34	0.027
serum albumin, g/L	38.88 ± 4.87	38.57 ±5.19	39.21 ± 4.48	0.16
serum creatinine, mg/dl	1.91 ± 1.79	2.08 ± 1.80	1.74 ± 1.76	<0.05
serum cystatin C, mg/L	1.87 ± 1.13	2.04 ± 1.20	1.70 ± 1.01	0.001
mGFR, ml/min/1.73 m^2	49.02 ± 22.45	46.17 ± 23.30	51.99 ± 22.28	0.005
eGFR, ml/min/1.73 m^2				
CKD-EPIcr	49.71 ± 26.08	45.48 ± 25.51	54.12 ± 26.0	<0.001
CKD-EPIcys	45.67 ± 25.70	41.49 ± 24.74	50.02 ± 26.01	<0.001
CKD-EPIcr-cys	47.01 ± 25.53	42.74 ± 24.74	51.47 ± 25.63	<0.001

*Data are presented as means and standard deviations, and counts (n) and percentages (%).
BMI, body mass index; mGFR, measured glomerular filtration rate; eGFR, estimated glomerular filtration rate; CKD-EPI, Chronic Kidney Disease Epidemiology.

TABLE 2 Performance of the three equations in individuals with and without diabetes.

	Bias	Precision	Accuracy	
	Median	IQR (P25, P75)	P30	RMSE
Overall ($n = 476$) CKD-EPIcr	−0.81	18.75 (−9.78, 8.97)	66.81	0.176
CKD-EPIcys	3.91[a]	18.63 (−5.75,12.88)	64.91	0.172
CKD-EPIcr-cys	2.24[a,b]	17.75 (−6.57,11.18)	71.01[a,b]	0.167
Individuals with diabetes ($n = 243$) CKD-EPIcr	1.26*	18.88 (−8.33, 10.55)	62.55*	0.199
CKD-EPIcys	5.51[a]*	18.51 (−4.02, 14.49)	60.08*	0.194
CKD-EPIcr-cys	4.08[a,b]*	17.27 (−4.59, 12.68)	63.78*	0.193
Individuals without diabetes ($n = 233$) CKD-EPIcr	−1.22	16.99 (−10.93, 6.06)	71.24	0.147
CKD-EPIcys	2.90[a]	17.82 (−6.96, 10.86)	69.96	0.145
CKD-EPIcr-cys	0.41[a,b]	15.49 (−7.10, 8.39)	78.54[a,b]	0.135

P30 represents the proportion of estimated glomerular filtration rate (GFR) within 30% of measured GFR.
IQR, interquartile range; RMSE, root mean square error, the square root of (log measured GFR – log of estimated GFR)2; CKD-EPI, Chronic Kidney Disease Epidemiology.
[a]$p < 0.05$ vs. CKD-EPIcr in the same group of individuals.
[b]$p < 0.05$ vs. CKD-EPIcys in the same group of individuals.
*$p < 0.05$ vs. corresponding equations used in individuals without diabetes.

In individuals without diabetes, the CKD-EPIcr-cys equation showed the lowest bias, and the highest precision and accuracy. The biases of CKD-EPIcr and CKD-EPIcr-cys were −1.22 and 0.41 ml/min/1.73 m^2, respectively, which were significantly different. Meanwhile, CKD-EPIcys had higher bias than CKD-EPIcr (2.9 vs. −1.22 ml/min/1.73 m^2, respectively, $p < 0.05$). Precision IQR showed that CKD-EPIcr-cys had the highest precision (15.49) and CKD-EPIcys had the lowest precision (17.82). With regard to accuracy, CKD-EPIcr-cys had higher P30 (78.54%) and lower RMSE (0.135) than each of CKD-EPIcr (71.24% and 0.147, respectively) and CKD-EPIcys (69.96% and 0.145, respectively), although the differences were not significant.

Comparison of the performance of equations between individuals with and without diabetes

As shown in Table 2, the performance of CKD-EPIcr-cys was less accurate in the diabetic group than in the non-diabetic group. Regarding bias, the median bias of CKD-EPIcr-cys in the diabetic group was significantly higher than in the non-diabetic group (4.08 vs. 0.41 ml/min/1.73 m^2, respectively, $p < 0.05$). Regarding precision, CKD-EPIcr-cys had the lower in the diabetic group than in non-diabetic group (17.27 vs. 15.49 ml/min/1.73 m^2, respectively). Regarding accuracy, CKD-EPIcr-cys was less accurate in the diabetic group; it had the lower P30 and higher RMSE in the diabetic group than in the non-diabetic group (P30, 63.78% vs.78.54%; RMSE, 0.193

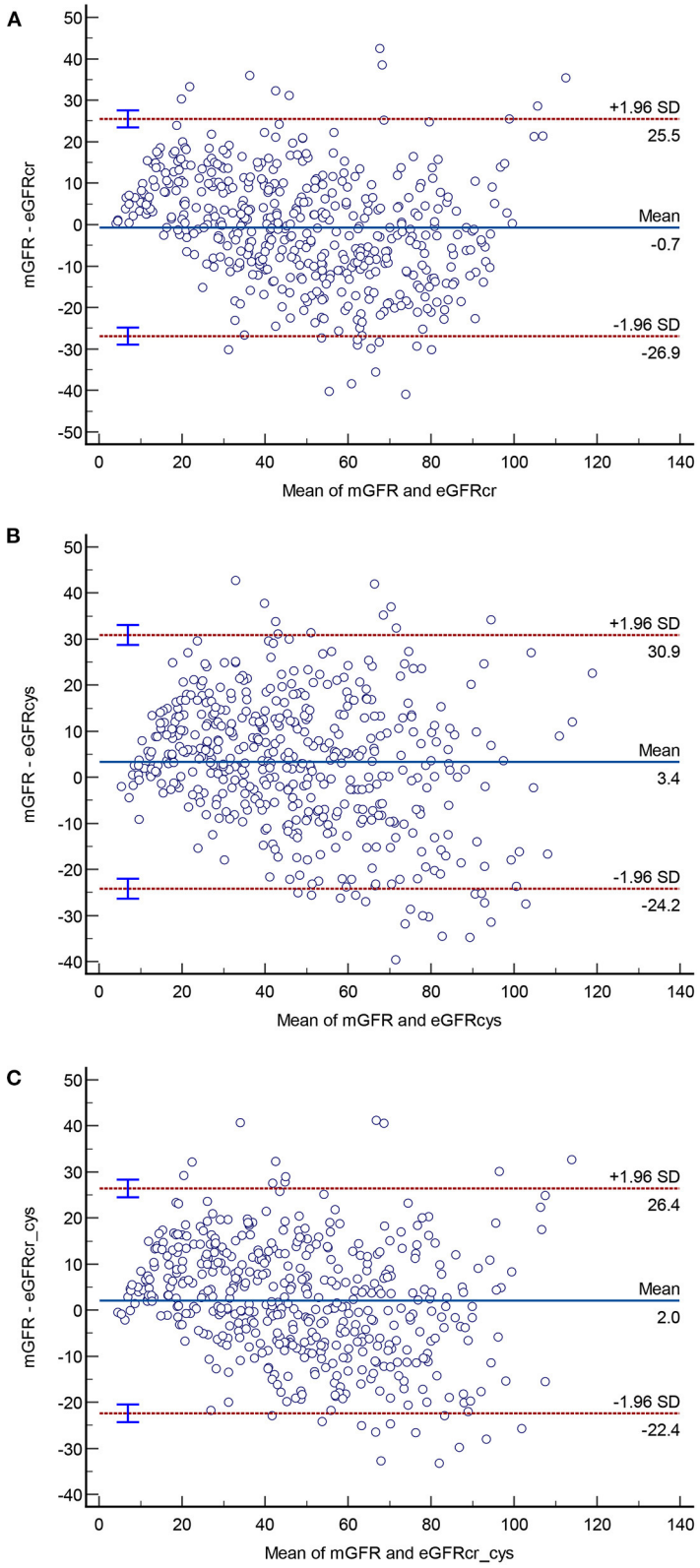

FIGURE 2
Bland-Altman plots of the three equations **(A–C)** in the overall population.

vs. 0.135). Similar results were observed for CKD-EPIcr and CKD-EPIcys equations.

Bland-Altman plots of the three equations compared to mGFR

Bland-Altman plots of the three equations in the overall population and in persons stratified by diabetes were presented in Figures 2, 3. In the overall population, Bland-Altman analysis showed that CKD-EPIcr-cys had the best agreement; it had the lowest gap between the 95% LOA (CKD-EPIcr, 52.4 ml/min/1.73 m^2; CKD-EPIcys, 55.1 ml/min/1.73 m^2; CKD-EPIcr-cys, 48.8 ml/min/1.73 m^2) (Figures 2A–C).

As shown in Figures 3A–F, in participants with and without diabetes, the gaps between the 95% LOA of the three equations were higher in the diabetic group than in the non-diabetic group (CKD-EPIcr, 53 vs. 51.3 ml/min/1.73 m^2, respectively; CKD-EPIcys, 55 vs. 54.8 ml/min/1.73 m^2, respectively; CKD-EPIcr-cys, 49.3 vs. 47.8 ml/min/1.73 m^2, respectively), suggesting that the consistency of these equations is lower in older subjects with diabetes than in their non-diabetic counterparts.

Discussion

We evaluated the performance of three CKD-EPI equations in a group of older adults with type 2 diabetes in comparison with non-diabetic counterparts. This study found that the CKD-EPI equations were less reliable in estimating GFR in older adults with type 2 diabetes than in the non-diabetic group. In addition, CKD-EPIcr-cys had the least bias and the best precision and accuracy in adults without diabetes. However, there seemed to be no performance advantages in using any of these equations in diabetic counterparts, although the median bias of CKD-EPIcr was relatively small.

In actual clinical practice, determination of GFR is an important step in assessing renal function. The ADA recommends annual screening for diabetic kidney disease by assessing urinary albumin excretion and GFR (17). The modification of diet in renal disease (MDRD) equation, which was developed in CKD patients, tends to be less accurate than CKD-EPI in those with GFR $\geq$ 60 ml/min/1.73 m^2 (11, 18). Thus, other equations were developed, such as CKD-EPI equation. The Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend use of the CKD-EPI equations to estimate GFR in adults of any age (19), which were developed in a North American and European population (11, 12). In addition to age and sex, these equations also take race into account. Although the proportion of patients aged $\geq$ 65 years with the CKD-EPI development data sets was 13%, previous study has found that CKD-EPI works satisfactorily in older adults with varying levels of GFR (20).

Older adults typically show a decrease in GFR, and this group is increasing in importance due to the gradual aging of the population (1). However, there have been few studies regarding

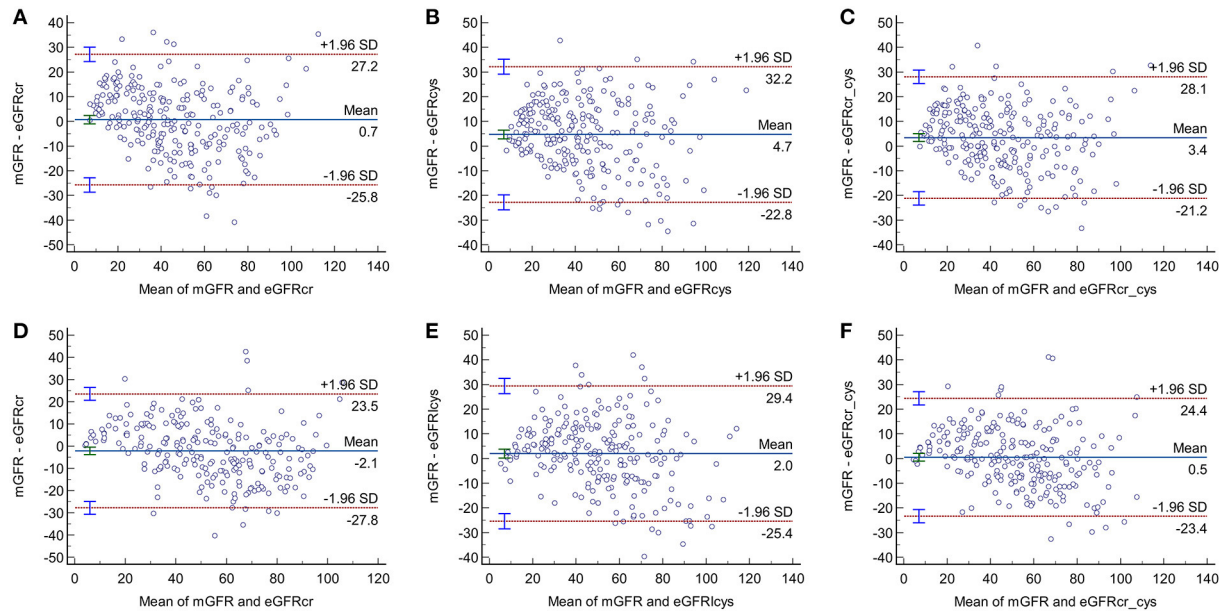

FIGURE 3
Bland-Altman plots of the three equations in older adults with **(A–C)** and without diabetes **(D–F)**.

the application of CKD-EPI equations in adults aged ≥ 65 years (6, 21, 22). As diabetes can induce renal damage and decrease GFR, it is necessary to clarify whether the CKD-EPI equations are equally applicable in older Chinese adults with and without type 2 diabetes.

In this study, GFR was measured using ^{99m}Tc-DTPA renal dynamic imaging, which was proposed by the Nephrology Committee of the Society of Nuclear Medicine (9). This method has been widely accepted as applicable for clinical evaluation of renal function (23, 24). Therefore, GFR obtained by ^{99m}Tc-DTPA renal dynamic imaging in this study was chosen as the reference GFR.

As muscle mass is frequently reduced in older adults, while plasma cystatin C is less affected, we explored the performance of CKD-EPI equations based on cystatin C alone and in combination with creatinine in older individuals with and without diabetes. All three equations showed a clinically poorer performance, with greater degrees of bias, lower precision, and lower accuracy, in older adults with diabetes than in non-diabetic controls. Our results were similar to a previous study by Camago et al. (25) in a population of 56 adult patients with type 2 diabetes and 55 healthy volunteers in whom the CKD-EPIcr equation was shown to be less accurate in the diabetic group compared to the non-diabetic controls. In a previous study, in a population of 215 diabetic and 192 non-diabetic CKD patients with a broad range of ages, Xie et al. (26) reported that CKD-EPIcr-cys showed the best performance among the CKD-EPI equations, and that eGFR equations were less accurate in the diabetic group than in the non-diabetic group. As these studies did not specifically focus on older adults, we then investigated four creatinine-based equations in people aged ≥ 65 years and our results suggested that the accuracy of creatinine-based GFR-estimating equations was lower in individuals with diabetes (21). However, further investigations are required to determine whether addition of diabetes can improve the performance of CKD-EPI equations in the older population.

On the other hand, the P30 of all three equations in the study failed to reach 80% in the elderly with or without diabetes, suggesting that these equations have limitations regarding accuracy in these populations. P30 exceeding 90% indicates that the equation meets the requirements for clinical interpretation (6, 15, 16). However, care is required in interpreting P30 decline in older adults, as small errors may still indicate inconsistent equations in those with low GFR (mean mGFR < 60 ml/min/1.73 m^2).

In addition, CKD-EPIcr overestimated GFR and CKD-EPIcys underestimated GFR whether in the overall population or in elderly subjects without diabetes. It may be caused by non-GFR determinants. Lower serum creatinine levels in older adults are often due to lower muscle mass and reduced protein intake. This is because lower muscle mass and reduced protein intake in older adults may lead to a decrease in serum creatinine levels, and the inflammatory status may lead to increased serum cystatin C levels. And in people with diabetes, all equations overestimated GFR and had greater biases, which appeared to be affected by glucose levels, although not fully explainable. Another misconception about the source of serum creatinine may be that a higher body mass index in the diabetic group would be an indicator of higher muscle mass. In fact, it indicates body fat buildup, not muscle mass (27).

This study had several limitations, as the sample size was relatively small sample size and from a single institution. Therefore, further studies with larger populations are required to verify our findings. In this study, GFR was measured by ^{99m}Tc-DTPA renal dynamic imaging, and not by inulin clearance. As inulin requires continuous infusions and repeated blood collection, it is not typically used in clinical settings. Finally, since this study is retrospective, some non-GFR determinants (e.g., C-reactive proteins) are incomplete.

Conclusion

In summary, the CKD-EPI equations were less reliable in estimating GFR in older adults with type 2 diabetes than in their non-diabetic counterparts. In adults with type 2 diabetes, there seemed to be no performance advantages in the use of any of these equations, albeit CKD-EPIcr had the least bias. However, in non-diabetic people, CKD-EPIcr-cys achieved optimal performance among the three equations. Nevertheless, there were still limitations regarding accuracy regardless of the presence or absence of type 2 diabetes. In older adults, especially in those with diabetes, early referrals for CKD treatment may decrease mortality, hospitalization rates, and rates of catheter use for dialysis.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of China-Japan Friendship Hospital. Written informed

consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

SJ contributed to conception and design of the study, and wrote the first draft of the manuscript. SJ and DZ collected the clinical data. WL revised the final version and was the guarantor of this work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Research progress on ferroptosis in diabetic kidney disease

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Ferroptosis is a newly discovered form of cell death that differs from other forms of regulated cell death at morphological, biochemical, and genetic levels, and is characterized by iron-dependent accumulation of lipid peroxides. Ferroptosis is closely related to intracellular metabolism of amino acids, lipids, and iron. Hence, its regulation may facilitate disease intervention and treatment. Diabetic kidney disease is one of the most serious complications of diabetes, which leads to serious psychological and economic burdens to patients and society when it progresses to end-stage renal disease. At present, there is no effective treatment for diabetic kidney disease. Ferroptosis has been recently identified in animal models of diabetic kidney disease. Herein, we systematically reviewed the regulatory mechanism of ferroptosis, its association with different forms of cell death, summarized its relationship with diabetic kidney disease, and explored its regulation to intervene with the progression of diabetic kidney disease or as a treatment.

KEYWORDS

iron-dependent cell death, iron metabolism, diabetic kidney disease, lipid peroxidation, diabetes mellitus

Introduction

The number of diabetic patients worldwide has more than doubled during the past 20 years (1). About 30-40% of these patients can develop diabetic kidney disease (DKD), of which about 50% can progress to end-stage renal disease (ESRD). DKD is the most common cause of ESRD, and is associated with increased incidence and mortality in diabetic patients. Timely diagnosis and treatment can delay its progression. However, a study by US scholars in 2021 showed that about 50% of patients with type 2 diabetes in CKD 3 stage are undiagnosed (2). In addition, there are differences between the animal models used in the preclinical study of DKD and the clinical studies in terms of age, renal function at the onset of the disease, and combination drugs, which leads to poor predictive value of animal experiments on the results of clinical trials, which increases the difficulty of treatment, so the control of DKD progression is not ideal (3). Current

treatments for DKD include controlling blood pressure and blood glucose, and the use of drugs that inhibit the renin-angiotensin system. However, these methods have limited effectiveness in preventing DKD progression. Therefore, deeper understanding of the underlying molecular mechanisms of DKD is needed to develop better therapies. Long-term high blood glucose level induces the expression of advanced glycation end-products, cytokines, growth factors, etc., activates signal transduction pathways, and promotes inflammation, endoplasmic reticulum stress, oxidative stress, mitochondrial dysfunction, and expression of autophagy-related genes, which constitute the main pathogenesis of DKD (4, 5). Ferroptosis is defined as iron-dependent regulated cell death, involving regulation at gene and protein levels, and is associated with abnormal accumulation of lipid reactive oxygen species (ROS), resulting in oxidative stress and cell death (6). Ferroptosis is ubiquitous in the body, and is involved in various physiological and pathological processes. In the pathogenesis of type 2 diabetes, ferroptosis not only leads to insulin secretion disorder, β-cell damage, endoplasmic reticulum stress, and production of ROS but also participates in the development of diabetes-related complications (7). In this review, we discussed the specific mechanism of ferroptosis and its role in DKD.

Discovery of ferroptosis

In 2003, Dolma et al. found that a novel compound erastin, selectively kills cancer cells differentially expressing RAS compared to other cells. In 2012, Dixon et al. investigated the mechanism by which erastin kills cancer cells using RAS mutations, and formally named this cell death process as "ferroptosis" (8). There are no morphological changes in the cell membrane and chromatin during ferroptosis, which are mainly manifested as decreased mitochondrial volume and mitochondrial crest, and increased mitochondrial membrane density (9). Biochemically, ferroptosis mainly manifests as declined glutathione peroxidase-4 activity, depletion of intracellular glutathione, and increased ROS level. Iron accumulation, glutathione depletion, and lipid peroxidation are indispensable and occur simultaneously during ferroptosis (10).

Association between ferroptosis and other forms of cell death

Ferroptosis was previously thought to be genetically and biologically different from other forms of cell death, but has been subsequently proven to share a common pathway with these forms (1). Apoptosis: It is now known that reactive oxygen species-induced lipid peroxidation plays an important role in apoptosis, mainly manifesting as lipid peroxidation products, which can be combined with extracellular signal-modulating kinase, p38 and other complexes to activate mitogen-activated protein kinase (MAPK) to activate caspase signal to initiate apoptosis. In addition, protein kinase C (PKC) can also be activated to amplify the apoptosis cascade. Since ferroptosis is accompanied by the formation of ROS and lipid peroxidation products, whether interfering with ferroptosis to reduce the production of ROS and subsequent lipid peroxidation plays a regulatory role in apoptosis needs to be further studied (11). Moreover, a recent study has indicated that ferroptosis-induced endoplasmic reticulum stress is associated with apoptosis. Protein kinase RNA-like endoplasmic kinase (PERK) - eukaryotic initiator 2α (EIF2α) - activating transcription factor 4 (ATF4) pathway-mediated endoplasmic reticulum stress is involved in regulation of enhancer binding protein (C/EBP[CCAAT-enhancer-binding protein] homologous protein] homologous protein, CHOP) and other target genes. Previous studies have shown that CHOP binds to the promoter of the pro-apoptotic protein p53-upregulated apoptotic factor (PUMA) during endoplasmic reticulum stress and induces the expression of PUMA, while trace analysis data have shown that the ferroptosis inducer artesunate (ART) can induce AFT4-dependent gene CHOP expression. In summary, ferroptosis inducers may promote the expression of pro-apoptotic protein PUMA through the PERK-EIF2α-ATF4-CHOP pathway. Interestingly, ART does not induce the expression of other pro-apoptotic proteins such as BCL-2 to promote apoptosis, that is, ferroptosis inducers do not promote apoptosis, which suggests antagonism in the induction of ferroptosis and apoptosis. Further research is needed to understand the role of ferroptosis inducers in PUMA activation for apoptosis (12). Whether a synergistic effect exists between ferroptosis inhibitors and apoptosis remains unknown but is likely based on the common characteristics ROS production and lipid peroxidation. Future research on regulating ferroptosis intervention-related diseases needs to focus on the apoptosis signal transduction pathway. (2) Autophagy: Autophagy is an evolutionarily conserved lysosomal-dependent degradation pathway. Nuclear receptor coactivator 4 (NCOA4)-mediated ferritinophagy can lead to ferroptosis by providing available labile iron. Lipid peroxides in ferroptosis induce autophagy by inhibiting adenosine monophosphate-activated protein kinase (AMPK) activation of mammalian target of rapamycin (mTOR), while knockout of autophagy-related genes such as *Atg5* and *Atg7* can reduce lipid peroxidation and intracellular Fe^{2+} inhibition of ferroptosis (11, 13). Autophagy, lipid peroxides, and ferroptosis involve complex interactions. Inhibition of ferritinophagy can interrupt ferroptosis in metabolic diseases. For example, NCOA4 knockout inhibits erastin-induced ferroptosis, while NCOA4 overexpression may be sufficient for ferroptosis. It is necessary to study the molecular regulatory mechanism of ferritinophagy in diseases (14). A new ferroptosis

inhibitor 9a can act on NCOA4 to ameliorate ischemic-reperfusion injury of the nervous system *via* the ferroptosis regulatory pathway, suggesting that NCOA4 is a promising drug target (15). In summary, apoptosis, autophagy and ferroptosis are closely linked by lipid peroxides. Different forms of cell death have unique morphological and biochemical characteristics, but there are some crosstalks between the regulators and components of these processes that jointly regulate cell death, and complete understanding of the interactions between the above processes can provide new insights on ferroptosis-related diseases.

Regulatory mechanism of ferroptosis

Induction of ferroptosis

It is currently believed that the main mechanism of ferroptosis is the catalytic lipid peroxidation of highly expressed unsaturated fatty acids on the cell membrane under the action of unstable Fe^{2+} or lipoxygenase, thereby inducing cell death. In addition, ferroptosis is also manifested by the reduction of the core enzyme GPX4 of the antioxidant glutathione system (1). Role of active iron: Under normal circumstances, the iron entering the body is bound with transferrin in the form of Fe^{3+}, enters the cell *via* transferrin receptor 1 on the cell membrane, and is reduced to Fe^{2+} by six-transmembrane epithelial antigen of the prostate 3 (STEAP3). The majority of iron is stored in the form of ferritin, and the minority is transported to a labile iron pool (LIP) in the cytoplasm *via* divalent metal transporter 1 (16). The intracellular iron output is mainly mediated by ferroportin (FPN), the main reason is that during iron overload in the body, a large amount of free iron in the LIP in the cells, i.e., Fe^{2+}, can provide hydroxyl radicals through the Fenton reaction and participate in lipid peroxidation, resulting in ferroptosis. The use of iron chelating agents (deferoxamine) to inhibit ferroptosis corroborates the role of iron overload in the process of ferroptosis, so ferroptosis is closely related to the steady state of iron metabolism in the body. Hepcidin can regulate FPN expression and affect the iron level in the system, while iron reaction elements (IREs)/iron regulatory proteins 1, 2 (IRP1, IRP2) regulate iron homeostasis at the cellular level (17). The imbalance of iron intake, storage, utilization, and outflow in the body affects the sensitivity of cells to ferroptosis. In addition to common iron transporters, heat shock proteins, contain iron enzymes such as heme oxygenase-1 (HO-1), which regulate ferroptosis through other pathways such as lipid peroxidation (18). (2) Main processes of lipid peroxidation: The peroxidation of polyunsaturated fatty acids on the cell membrane is the key process of ferroptosis. Polyunsaturated fatty acids first form PE-PUFA under the actions of Acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPACT3), followed by formation of PE-PUFA-OOH through the action of lipoxygenase or non-enzymatically through autoxidation, which ultimately causes cell death (19). Lipid peroxidation mainly occurs in two ways: non-enzymatic radical chain reaction and enzyme catalytic occurrence, non-enzymatic radical chain reaction is mainly mediated by iron provided by hydroxyl radical through Fenton reaction, and enzyme catalytic reaction is mediated by the aloxygenases (ALOXs), especially ALOX15. Lipoxygenase was initially considered as an important driving factor of lipid peroxidation, but its low expression in some cancer cells suggested that other enzymes may mediate lipid peroxidation. Koppula et al. summarized previous studies and concluded that cytochrome P450 reductase transfers electrons from NADPH to oxygen to produce hydrogen peroxide, thereby driving lipid peroxidation, membrane rupture and ferroptosis (20). Therefore, ALOXs may not be necessary for ferroptosis, and may also function in some more complex environments or situations by supplementing the auto-oxidation pathway, which needs to be further studied. Many hypotheses have been proposed on the mechanism by which lipid peroxides cause ferroptosis, including changes in cell membrane structure and permeability affecting cell survival. Lipid peroxides that can break down to produce toxic derivatives such as malondialdehyde (MDA), causing DNA and protein damage. In addition, once lipid peroxides are formed, they may further amplify ROS signaling and drive the mitochondrial cysteine protease signaling pathway, linked to pyroptosis (19). The substrates of ferroptosis are mainly polyunsaturated fatty acids. Recent studies have found that long-chain saturated fatty acids also participate in ferroptosis, but the mechanism remains unclear. Through endogenous metabolites and genome-wide CRISPR screening, Cui et al. confirmed that peroxisomal fatty acyl-CoA reductase 1 (FAR1) is a key factor in ferroptosis mediated by long-chain saturated fatty acids (21). Interestingly, a recent study also reported that exogenous monounsaturated fatty acids suppress ferroptosis requiring acyl-CoA synthetase long-chain family member 3 (ACSL3), which is related to the inhibition of lipid ROS accumulation and reduction of phospholipid levels of oxidizable polyunsaturated fatty acids (22). Thus, the activation of ACSL4 rather than other homologous enzymes is necessary for lipid peroxidation, and ACSL4 expression can regulate the occurrence of ferroptosis. (3) The collapse of the antioxidant system: 1) Xc^-system-GSH-GPX4: GPX4 is a key defense mechanism to prevent cellular ferroptosis, with selenocysteine in its active center. It reduces toxic lipid hydroperoxides to corresponding hydroxyl derivatives to inhibit ferroptosis *via* intracellular glutathione (23). Lipid peroxidation and subsequent ferroptosis were observed in mice with GPX4 conditional knockout, indicating that GPX4 is a key regulator of ferroptosis (24). GSH acts as an electron donor during GPX4 involvement in ferroptosis, so regulation of the GSH axis is

necessary to maintain GPX4 activity. Cystine, one of the components of GSH, is the main limiting process of GSH synthesis. It is transferred into cells by the cystine/glutamate reverse transporter (Xc^-system), which is composed of SLC7A11 and SLC3A2. Inhibition of this system can lead to depletion of cysteine in cells, and induce ferroptosis (25). The nuclear factor erythroid-related factor 2 (Nrf2) affects SLC7A11 expression to resist ferroptosis, and p53 protein also regulates its expression to affect cysteine intake, thereby blocking GSH synthesis and inducing ferroptosis (26). The active center of GSH contains selenocysteine, so the regulation of the selenium axis also affects the activity of GPX4. The mechanism of synthesis of GPX4 is not well understood. Zhang et al. found that cystine and cysteine promote GPX4 protein synthesis by activating rapamycin complex 1 (mTORC1), and its inactivation sensitizes cancer cells to ferroptosis by reducing GPX4 (27). These results suggested that the synthesis of GSH can be regulated by regulating the Xc^-system to affect ferroptosis. In addition, some cells can also synthesize cysteine from methionine *via* the transsulfuration pathway, which resists ferroptosis to some extent (28). Glutamate-cysteine ligase activity has been found to prevent the accumulation of glutamate in cells under cysteine deficiency, thereby preventing ferroptosis in non-small cell lung cancer (29). 2) Nuclear factor erythroid-related factor 2 signal pathway: NRF2 is involved in constituting and controlling defense pathways for oxidative stress and may play a role in regulating ferroptosis, given that many proteins stored and transported with iron are controlled by NRF2, which also affects enzymes associated with GSH synthesis as described above, so targeting NRF2 to regulate lipid peroxidation and ferroptosis is a viable disease intervention strategy (30, 31). Therefore, GPX4 inactivation is not the only condition for ferroptosis, and the collapse of many antioxidant mechanisms in the body leads to the occurrence of ferroptosis. In summary, ferroptosis is the result of a comprehensive process, which requires the high expression of lipid-promoting peroxidases such as ACSL4, the role of active iron, and the collapse of antioxidant systems such as GPX4. The key processes involved in ferroptosis are shown in Figure 1.

Inhibition of ferroptosis

Numerous mechanisms in the body inhibit ferroptosis: (1) NADPH-FSP1-coenzyme Q10 pathway: GPX4 is considered as a major antioxidant of ferroptosis. Different cancer cells were found to have different sensitivities to GPX4 inhibitors, and it was speculated that there were other factors controlling resistance to ferroptosis. In 2019, ferroptosis suppressor protein 1 (FSP1) was identified as an important antioxidant protein, which acts through coenzyme Q10 (ubiquinone). The reduced form of ubiquinone can capture active free radicals and

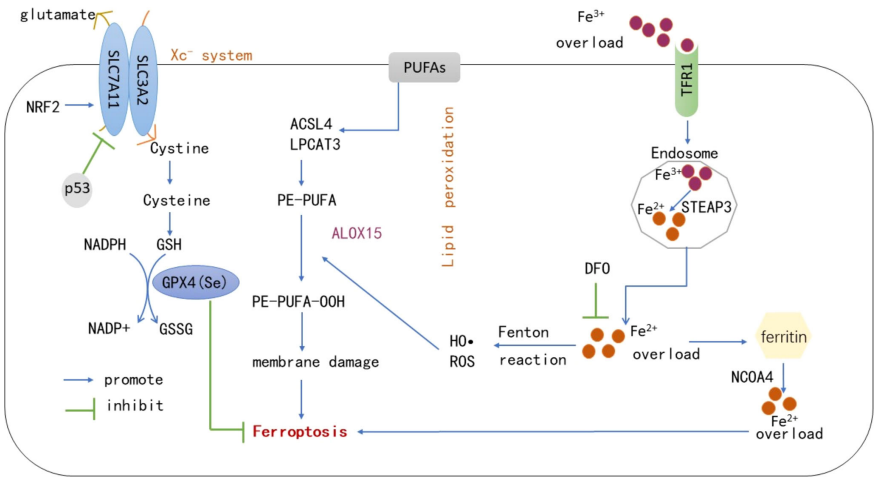

FIGURE 1
Under the actions of ACSL4, LPACT3 and ALOX15, PUFAs on the cell membrane form PE-PUFA-OOH. Under excessive iron conditions, some iron is stored in the form of ferritin, and the remaining free Fe^{2+} generates numerous ROS and hydroxyl radicals through the Fenton reaction, which induces ferroptosis on the cell membrane. However, GPX4 reduces PE-PUFA-OOH to -OH and inhibits ferroptosis *via* the effect of GSH. The synthesis of GSH is mainly regulated by the Xc^-system, through which cystine transported into cells is reduced to cysteine to synthesize GSH. p53 can inhibit SLC7A11 expression in this system and promote ferroptosis. In addition, NRF2 can affect the expression of SLC7A11 against ferroptosis. NCOA4-mediated ferritinophagy leads to ferritin production by providing labile iron, deferoxamine can also inhibit ferroptosis by chelating active iron. PUFAs, Polyunsaturated fatty acids; GPX4, Glutathione peroxidase 4; GSH, Reduced glutathione; GSSH, Oxidized glutathione; ACSL4, Acyl-CoA synthetase long-chain family member 4; LPACT3, Lysophosphatidylcholine Acyltransferase 3; ALOX15, Arachidonic acid 15-lipoxygenase; NCOA4, Nuclear receptor coactivator-4; ROS, Reactive oxygen species; TFR1, Transferrin receptor 1; NRF2, Nuclear factor erythroid-related factor 2; DFO, Deferoxamine; STEAP3, six-transmembrane epithelial antigen of the prostate 3.

reduce the production of intracellular lipid peroxides. Moreover, FSP1 can catalyze the regeneration of coenzyme Q10 through NADPH (32). As the main effector of the FSP1 pathway, supplementation with selenium and coenzyme Q10 has been reported to reduce cardiovascular mortality in the elderly (33). In summary, NADPH-FSP1-CoQ10 exists as a parallel system independent of GPX4 action, and inhibition of FSP1 may be an effective strategy to promote death of cancer cells and other diseases. (2) GCH-1-BH4: Tetrahydrobiopterin has antioxidant effects *in vitro*, and its role in regulating ferroptosis has been recently clarified. Tetrahydrobiopterin can reduce lipid peroxidation by producing coenzyme Q10 to reduce oxidative damage and cause lipid remodeling (34). GTP cyclohydrolase 1 (GCH-1) is a rate-limiting enzyme regulating the synthesis of tetrahydrobiopterin. Overexpression of GCH-1 has a protective effect on RSL3-induced ferroptosis, but not on apoptotic inducers, indicating that GCH-1 selectively protects cells against ferroptosis (35) (3). Post-translational modifications (PTMs): PTMs include phosphorylation, acetylation, methylation, etc. Most PTMs are reversible. PTMs not only diversify the function of proteins but also enable cells or organisms to respond quickly and strictly to stress. The role of PTMs in ferroptosis has gradually become a research hotspot in recent years (35). Recent studies on tumor cells reported that when cells are hungry, energy stress activates the AMP-activated protein kinase (AMPK), activates of acetyl-CoA carboxylase (ACC) phosphorylation, and further inhibits the synthesis of polyunsaturated fatty acids and ferroptosis. Activating this process has been found to prevent renal ischemia-reperfusion damage. AMPK can promote ferroptosis by inhibiting the transport of SLC7A11-mediated cystine, although its role in ferroptosis remains controversial. Thus, AMPK and ferroptosis need further study (35–37). The role of AMPK in ferroptosis is related to phosphorylation. There are few studies on how other PTMs are involved in the regulation of ferroptosis. Elucidating PTMs associated with inhibition of ferroptosis under different conditions is an interesting research direction for the future.

Ferroptosis and DKD

With the increase in global prevalence of diabetes, the prevalence of chronic kidney disease caused by type 2 diabetes has increased from 1.39% in 1999 to 1.52% in 2009 and 1.74% in 2019 (38). Typically, proteinuria is considered as a biomarker of DKD, preceding the loss of renal function. However, a subset of patients have no proteinuria but develop loss of renal function, which is also known as non-proteinuria diabetic nephropathy, which indicates that DKD has clinical heterogeneity and increases the difficulty of treatment (39). The pathogenesis of traditional DKD is believed to be caused by changes in renal hemodynamics (high stress, high filtration, high perfusion), increased oxidative stress caused by ischemia and abnormal glucose metabolism, inflammation, and hyperactivity of the renin-angiotensin-aldosterone system. Recent molecular and cellular studies have continued to explore new areas of DKD pathogenesis, including genetic and epigenetic modifications, podocyte autophagy, and mitochondrial dysfunction, providing more possible directions for the treatment of DKD (40). The role of ferroptosis was first identified in renal ischemic-reperfusion injury, and there are limited studies on DKD. Previous studies found that iron-chelating agents could delay the progression of DKD, the underlying mechanism may be that iron chelating agents exert a protective renal effect by reducing oxidative stress, inflammation, and tubular interstitial fibrosis. However, the exact mechanism by which excessive iron promotes DKD progression remains unclear (41, 42). Because ferroptosis process is accompanied by excessive lipid ROS production, which can lead to oxidative stress, and kidney-rich mitochondrial structure is more vulnerable to oxidative stress damage, the traditional pathogenesis of DKD is also involved in oxidative stress, which suggests that ferroptosis may be associated with DKD. Many scholars have also aimed to explore new ways to control the progression of DKD based on this perspective. The mechanism of ferroptosis in DKD was initially studied mainly at the cellular and animal levels. For example, Wang et al. explored the role of ferroptosis in the progression of DKD using *in vivo* and *in vitro* experiments, and found that ferroptosis-related protein GPX4 expression was decreased, ACSL4 expression was increased, and lipid peroxide products and iron content were also increased in mouse models of DKD (43), which was similar to the results of Li et al. who also found that Nrf2 levels were decreased in the DKD animal model, which inhibited ferroptosis by upregulating Nrf2 through fenofibrate therapy and delayed the progression of DKD in mice (41), revealing the development mechanism of DKD from a new perspective. As mentioned above, the occurrence of ferroptosis is related to the NRF2 signaling pathway. Therefore, Li's research links ferroptosis more closely with DKD. Subsequently, Kim et al. also reported that ferroptosis was associated with DKD. They evaluated changes in ferroptosis-related molecules in renal biopsy tissues of patients with DKD, and found that SLC7A11 and GPX4 mRNA expression was reduced in renal tubules (44). The above studies have confirmed that ferroptosis is associated with DKD, but the mechanism is unclear. Feng et al. found that ferroptosis can damage renal tubules through hypoxia-inducible factor-1α (HIF-1α)/heme oxygenase (HO-1) pathway, while the selective ferroptosis inhibitor Ferostatin-1 (Fer-1) treatment inhibits the expression of HIF-1α and HO-1, and reduces tubular damage and fibrosis in diabetic mice by reducing tubular iron overload, inhibiting ROS formation, oxidative stress, and lipid peroxidation (45). The association between ferroptosis and DKD was also studied at the clinical level. We found that the expression level of ferroptosis-related protein GPX4 was reduced in the serum of patients with DKD, while the

expression of ACSL4, PTGS2, HMGB1, ROS release and MDA generation were upregulated. The inhibition of HMGB1 was further found to promote the expression of Nrf2 to prevent glucose-induced mesangial cell ferroptosis and inhibit the inflammatory response. These findings provide new treatment strategies for DKD by HMGB1 and ferroptosis (46). High mobility group box 1 (HMGB1) is a typical damage-associated molecular pattern (DAMP), which are endogenous mediators causing inflammation, and can be released by apoptosis, ferroptosis, and necrosis (47). Once released, HMGB1 can further bind to receptors such as Toll-like receptor 4 (TLR4) and glycosylation end-product specific receptor (AGER) to mediate immune responses. Therefore, inhibition of HMGB1 release and extracellular activity is a potential anti-inflammatory strategy for the treatment of diseases such as DKD (48). In summary, the above studies have shown that ferroptosis also plays a pathological role in the development of DKD. The main studies on ferroptosis with DKD are listed in Table 1.

Regulation of ferroptosis for the treatment of DKD

Ferroptosis is related to the action of active iron, lipid peroxidation, and weakened antioxidant capacity, and intervention of these processes may inhibit ferroptosis for therapeutic purposes. Current evidence indicates the following: 1) The ACSL4 inhibitor rosiglitazone reduces renal pathological damage in DKD mice by reducing lipid peroxidation (43). 2) Iron chelating agents are also an effective method of inhibiting ferroptosis by reducing excess intracellular iron, since the occurrence of ferroptosis depends on excess intracellular iron producing large amounts of ROS through the Fenton reaction. Kim and Feng et al. have demonstrated that Fer-1 mitigates kidney damage in mice with DKD (44, 45). Since the regulation of ferroptosis is a multi-pathway, more targets can be identified for the prevention and control of DKD. We present several possibilities: 1) Reduce oxidative stress: the production of numerous ROS in cells and the subsequent generation of active free radicals are the key factors mediating lipid peroxidation, and the NADPH-FSP1-CoQ10 pathway in the regulatory mechanism of ferroptosis is independent of the antioxidant mechanism existing in GPX4, mainly by reducing free radicals. Further research is needed to inform whether vitamin E supplementation can inhibit ferroptosis and reduce kidney damage to some extent by capturing active free radicals. 2) NRF2 signaling pathway: NRF2 is known to be one of the defense pathways for oxidative stress *in vivo*, which can neutralize ROS, regulate enzymes involved in iron metabolism and GSH synthesis in ferroptosis. However, Nrf2 overactivation was found to induce ferroptosis through the HO-1 pathway in cancer (49), which is a double-edged sword, so it is necessary to further understand the relationship between the upstream and downstream regulation of Nrf2 and ferroptosis, and mechanisms of alteration of Nrf2 levels in physiological and pathological states. 3) Inflammatory pathway: DKD is widely regarded as a chronic inflammatory disease. The ferroptosis process is accompanied by DAMPs and inflammatory factors changes. DAMPs lead to renal inflammatory cell infiltration through the immune response, and release inflammatory factors, amplify the immune response, resulting in a sustained inflammatory response, related to the progression of DKD. Ferroptosis is complemented by inflammation. For example, in mouse models of nonalcoholic steatohepatitis, ferroptosis has been found to occur with the expression of pro-inflammatory factors such as tumor necrosis factor-α and IL-6, and treatment with deferoxidamine can significantly inhibit the progression of nonalcoholic steatohepatitis, manifested by decreased lipid peroxidation levels (50). In addition, some inflammatory cytokines have also been shown to affect the activity of GPX4 in cancer cells (51), and some anti-inflammatory drugs have been found to inhibit ferroptosis in some cellular models (52). These findings suggest that controlling inflammation during ferroptosis may have a wide range of regulatory effects with clinical benefits. 4) Protein post-translational modification: The changes of various protein activities in the process of ferroptosis may be related to PTMs. Identifying DKD-

TABLE 1 Mechanism and biochemical features of ferroptosis in DKD.

Cell/Animals/clinical	Mechanism	Biochemical features	Reference
Animal: STZ-induced diabetic mice and db/db mice Cell: NRK-52E cells and HK-2 cells	ACSL4 regulates ferroptosis	Increase in ACSL4 and MDA Increase in ACSL4	Wang Y, et al. (43)
Cell: NRK-52E cells Animal: STZ-induced diabetic mice Clinical: Kidney biopsy samples	N.A.	Decrease in xCT, GPX4 and GSH Increase in MDA, 4-HNE, iron and FTH1 Decrease in xCT and GPX4	Kim S, et al. (44)
Animal: db/db mice	HIF-1α/HO-1 pathway might be regulated ferroptosis	Decrease in GSH-Px, CAT, SOD Elevated ferritin, HIF-1α/HO-1 and increase in MDA	Feng XM, et al. (45)
Cell: Renal mesangial SV40-MES 13 cells Clinical: blood samples collected from DKD patients	HMGB1/Nrf2 regulates HG induced ferroptosis	Decreased GPX4 Increase in ROS, MDA, ACSL4, PTGS2 and LDH release	Wu Y, et al. (46)

N.A., GPX4 regulates ferroptosis.

specific biomarkers to activate or inactivate the protein modifications may more easily regulate ferroptosis for therapeutic purposes. Although the main mechanism of ferroptosis is currently well understood, deeper understanding is needed to provide a better theoretical basis for the treatment of DKD and other diseases.

Conclusion

When DKD progresses to end-stage renal disease, renal replacement therapy is the only option, which also increases the risk of death, so early intervention is the key. However, the previous drugs used to treat DKD (RAAS inhibitors) have not been found to prevent the occurrence of DKD. With the advances in the pathogenesis of DKD, new drugs for the prevention and treatment of DKD, such as endothelin receptor antagonists, protein kinase C inhibitors, phosphodiesterase inhibitors, etc., are being investigated. The discovery of ferroptosis provides a new approach. However, as mentioned earlier, although ferroptosis has different morphological and biochemical characteristics from apoptosis and autophagy, it is closely linked by lipid peroxidation, and the interaction between these processes should be noted when targeting ferroptosis in the treatment of DKD, whether it is synergistic or antagonistic. Notably, the association between ferroptosis and DKD is mainly verified at the cellular level and in animal models, without any clinical trials. However, understanding the specific mechanism of ferroptosis may provide a strategy for the treatment of DKD. Finally, ferroptosis-specific biomarkers can also be investigated in the future to detect the progression of DKD.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Urinary IL-18 is associated with arterial stiffness in patients with type 2 diabetes

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Objective: Diabetic kidney disease (DKD) has been shown to be associated with an excess risk of cardiovascular death. Inflammation has been considered central to type 2 diabetes (T2D) pathophysiology, and inflammation markers have been linked to cardiovascular disease. The serum and urinary IL-18 levels were significantly elevated in patients with T2D; however, whether interleukin 18 (IL-18) are associated with the severity of arterial stiffness remains to be determined. This study examined the relationship of IL-18 levels with pulse wave velocity (PWV) as a reflector for arterial stiffness in patients with T2D.

Methods: A total of 180 participants with T2D who had undergone PWV examination were enrolled. Serum and urinary IL-18 levels were measured using sandwich enzyme linked immunosorbent assay (ELISA) kits. Arterial stiffness was determined by carotid–femoral PWV (cf-PWV) and carotid–radial PWV (cr-PWV).

Results: The urinary IL-18 levels correlated positively with cf-PWV in patients with T2D with DKD (r = 0.418, p < 0.001); however, we found no significant correlation between urinary IL-18 and cf-PWV in diabetic subjects without DKD. In addition, we found no significant correlation between urinary IL-18 and cr-PWV in participants with T2D with or without DKD. Moreover, the association remained significant when controlling for arterial stiffness risk factors, urinary albumin-to-creatinine ratio and estimated glomerular filtration rate. cf-PWV was greater in the higher group of urinary IL-18 than in the lower group. Nevertheless, we found no significant correlation between serum IL-18 and cf-PWV in participants with T2D.

Conclusion: The urinary IL-18 levels appear to be associated with greater cf-PWV, suggesting the link between urinary IL-18 and arterial stiffness in patients with T2D.

KEYWORDS

IL-18, pulse wave velocity, arterial stiffness, diabetic kidney disease, inflammation

Introduction

The association between cardiovascular disease (CVD) and diabetes has been known for decades. Clinical and experimental studies have provided evidence and probable mechanisms that link diabetes to increased atherosclerosis. In particular, patients with T2D had more extensive atherosclerotic CVD (ASCVD) and more non-calcified plaques than patients with T1D. Further experimental studies are needed to elucidate these questions (1). Greater calcification in arterial media is also found in patients with diabetes compared with control subjects, especially T2DM (2). Diabetic kidney disease (DKD) has been shown to be associated with an excess risk of premature death and CVD death (3, 4). Despite this, the reasons for this relationship are incompletely understood.

The relationship of inflammation to insulin resistance is considered central to T2DM pathophysiology (5, 6). Markers of inflammation have been linked to CVD and CVD death. Clinical trials have overwhelmingly shown beneficial effect of targeting inflammation in prevention of the incidence of CVD in human with diabetes (7, 8). Moreover, systemic inhibition of nucleotide-binding oligomerization domain-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome was recently described to prevent increased atherosclerosis in mice with diabetes (9). IL-18 is a proinflammatory marker (10) and biomarker of kidney tubule injury and repair (11, 12). The serum and urinary IL-18 levels were significantly elevated in patients with T2D compared with control subjects (13). Elevated serum levels of IL-18 were associated with carotid intima-media thickness (13) and development of DKD in normal albuminuria subjects (14).

Unlike renal and retinal microvascular disease, there is no pathological fingerprint identifying a distinct atherosclerosis or arterial media calcification in the setting of diabetes. Pulse wave velocity (PWV) is assessed by measuring transit distance and transit time between two sites in the arterial system and taking their ratio. Carotid–femoral PWV (cf-PWV) is the current clinical gold standard measurement of arterial stiffness and has been established as a cardiovascular risk marker (15).

The aim of this study was to examine the relationship of IL-18 levels with PWV as a reflector for arterial stiffness and CVD.

Methods

Subjects

The study protocol was approved by Institutional Ethical Committee of Nanjing Medical University (approved No. 2019KY097), and written informed consent was obtained from all subjects. The study conforms to the principles outlined in the Declaration of Helsinki. A total of 180 subjects for this study were enrolled from patients with T2D of the Department of Internal Medicine at the Second Affiliated Hospital of Nanjing Medical University, who had undergone PWV examination between January 2020 and December 2020. Subjects were diagnosed as having T2D according to the WHO criteria and provided multiple morning urine and blood samples for assessment of urinary albumin-to-creatinine ratio (UACR) and estimated glomerular filtration rate (eGFR). On the basis of multiple UACR and eGFR measurements, subjects were classified as T2D without DKD (T2D − DKD, n = 115) or T2D with DKD (T2D + DKD, n = 65). Subjects with acute inflammatory diseases or malignant neoplasm were excluded, because the levels of inflammation can be markedly enhanced by such disease. Hypertension was defined as a blood pressure (BP) ≥ 130/80 mmHg or current use of antihypertensive medications.

Blood and urine examination

Each individual provided blood and morning spot urine samples at baseline for biochemical measurements. Samples were centrifuged at 3,000 rpm at 4°C for 15 min, and aliquots were stored at −80°C if not analyzed immediately. Serum and urinary IL-18 levels were measured using sandwich ELISA kits (DY318-05, R&D Systems). Urinary IL-18 was normalized by urinary creatinine (UCr). The intra- and inter-assay coefficient of variations (CVs) for IL-18 were both less than 10%. The sensitivity of the assay was 5.47 pg/ml. These parameters were measured twice for each individual, and geometric mean was used as the baseline value in the analysis.

Measurement of pulse wave velocity

cf-PWV and carotid–radial PWV (cr-PWV) were determined on a fasting state in the morning under room temperature (21°C–25°C) using the Complior Analyzer device (Artech Medical, Paris, France) according to the manufacturer's introduction. Coffee, tea, or nitrates were not allowed within 2 h, and long-acting nitrates were restrained for 12 h before measurement. An experienced technician from the Second Affiliated Hospital of Nanjing Medical University performed the test for all participants. Before measurement, patients rested for 10 min and had their BP measured using a validated oscillometric device (Omron HEM-7130: Omron Healthcare Co., Ltd., Kyoto, Japan). Three probes were placed in a place of palpable pulse of the carotid, femoral, and radial artery, respectively. Ten consecutive recordings were averaged to calculate the transit time using the intersecting tangent algorithm. Carotid–femoral and carotid–radial distances were calculated as direct measurements multiplied by 0.8. Any measurement with a tolerance value more than 3 ms was considered invalid.

Statistical analysis

All analyses were performed with SPSS 25 software package. Because the distribution of the IL-18 levels appeared to be left-skewed, they were normalized by log-transformation. Comparisons between groups were performed by using an unpaired Student's *t*-test for normally distributed variables and a Mann–Whitney *U*-test for non-normally distributed variables. Associations between IL-18 levels and characteristics of type 2 diabetes were examined by Pearson correlation analysis for continuous variables and by Spearman correlation test for categorical variables. Association between urinary IL-18 and cf-PWV was determined by a multivariable linear regression analysis with a stepwise backward method. Mean cf-PWV was compared across the median of the urinary IL-18 levels by the general linear model, followed by covariance analysis. A *p*-value of less than 0.05 was taken to be statistically significant.

Results

Clinical characteristics in Table 1 indicate that participants with type 2 diabetes had a median age of 56.00 (47.00, 60.00) years, and 66.1% were male patients. Furthermore, participants had mean body mass index (BMI) of 25.01 (22.95, 27.52) kg/m^2, HbA1c level of 8.30% (7.00%, 10.23%), and eGFR of 101.77 (92.47, 109.48) ml/min/1.73 m^2. A proportion had albuminuria (35.6%) and hypertension (40%).

According to the presence or absence of DKD, as described by UACR ≥ 30 mg/g and/or eGFR < 60 ml/min/1.73 m^2, the proportion of male sex, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), triglyceride, and serum creatinine were all significantly higher in the group of diabetic subjects with DKD than in the group without DKD. Subjects with DKD had significantly lower levels of albumin and high-density lipoprotein (HDL) compared with subjects without DKD. Interestingly, the urinary levels of IL-18 and cf-PWV were significantly elevated in subjects with DKD compared with those without DKD [UIL-18 193.24 (102.78, 257.81) vs. 128.28 (82.67, 204.14) pg/mg UCr, $p = 0.005$; cf-PWV 9.20 (7.50, 10.25) vs. 7.90 (6.80, 9.20), $p < 0.001$], whereas the serum IL-18 levels and cr-PWV were not different in the two groups.

By univariate linear regression analysis in Table 2, we found significant correlations between urinary IL-18 and age ($r = 0.224$, $p = 0.002$), male sex ($r = -0.253$, $p = 0.001$), duration of T2D ($r = 0.259$, $p < 0.001$), SBP ($r = 0.228$, $p = 0.002$), hemoglobin ($r = -0.239$, $p = 0.002$), albumin ($r = -0.305$, $p < 0.001$), eGFR ($r = -0.237$, $p < 0.001$), UACR ≥ 30 mg/g ($r = 0.224$, $p = 0.003$),

TABLE 1 Clinical characteristics of subjects with type 2 diabetes.

	Total (n = 180)	T2D − DKD (n = 115)	T2D + DKD (n = 65)	*p*
Age (years)	56.00 (47.00, 60.00)	56.00 (46.00, 60.00)	56.00 (47.00, 61.00)	ns
Male (n, %)	119, 66.1%	68, 59.1%	51, 78.5%	0.008
Duration of T2D (years)	4.79 (1.00, 10.72)	4.00 (1.00, 10.16)	5.00 (2.04, 13.87)	ns
BMI (kg/m^2)	25.01 (22.95, 27.52)	24.52 (22.32, 26.91)	25.65 (23.92, 28.03)	0.025
Hypertension (n, %)	72, 40%	37, 32.2%	35, 53.8%	0.004
SBP (mmHg)	134.06 ± 17.76	130.97 ± 16.20	139.54 ± 19.14	0.002
DBP (mmHg)	84.51 ± 10.59	82.33 ± 9.20	88.37 ± 11.81	0.001
FBG (mmol/L)	8.51 (6.88, 10.90)	8.51 (6.93, 10.90)	8.40 (6.84, 10.77)	ns
HbA1c (%)	8.30 (7.00, 10.23)	8.30 (7.00, 10.33)	8.30 (7.10, 10.07)	ns
Hb (g/L)	144.18 ± 17.21	143.67 ± 17.31	145.03 ± 17.14	ns
Albumin (g/L)	45.50 (41.35, 48.90)	46.90 (42.20, 49.30)	44.25 (39.78, 47.48)	0.007
TC (mmol/L)	4.66 (3.88, 5.40)	4.70 (3.98, 5.46)	4.63 (3.65, 5.26)	ns
TG (mmol/L)	1.69 (1.10, 2.64)	1.54 (1.03, 2.36)	2.02 (1.19, 2.82)	0.044
HDL (mmol/L)	1.05 (0.91, 1.27)	1.08 (0.95, 1.33)	1.00 (0.88, 1.19)	0.010
LDL (mmol/L)	2.97 (2.33, 3.63)	3.04 (2.38, 3.70)	2.81 (2.23, 3.34)	ns
Creatinine (μmol/L)	66.00 (54.35, 75.55)	63.80 (52.60, 72.00)	71.70 (59.50, 99.90)	<0.001
eGFR (ml/min/1.73 m^2)	101.77 (92.47, 109.48)	101.96 (96.51, 109.62)	97.37 (69.05, 108.38)	0.005
UACR ≥ 30 mg/g (n, %)	64, 35.6%	0	64, 98.5%	–
SIL-18 (pg/ml)	163.62 (117.63, 230.97)	166.76 (122.62, 231.96)	150.17 (109.05, 218.35)	ns
UIL-18 (pg/mg UCr)	140.82 (95.29, 227.40)	128.28 (82.67, 204.14)	193.24 (102.78, 257.81)	0.005
Cf-PWV (m/s)	8.30 (7.03, 9.70)	7.90 (6.80, 9.20)	9.20 (7.50, 10.25)	<0.001
Cr-PWV (m/s)	9.45 (8.43, 10.30)	9.30 (8.40, 10.20)	9.60 (8.60, 10.75)	ns

BMI, body mass index; cf-PWV, carotid–femoral pulse wave velocity; cr-PWV, carotid–radial pulse wave velocity; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FBG, fast blood glucose; HDL, high-density lipoprotein cholesterol; Hb, hemoglobin; HbA1c, hemoglobin A1c; LDL, low-density lipoprotein cholesterol; SBP, systolic blood pressure; SIL-18, serum interleukin-18; TC, total cholesterol; TG, triglyceride; T2D, type 2 diabetes; UACR, urinary albumin-to-creatinine ratio; UCr, urinary creatinine; UIL-18, urinary interleukin-18.

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and serum IL-18 ($r = 0.219$, $p = 0.004$) in participants with T2D. However, we found no significant correlation between urinary IL-18 and BMI, DBP, fast blood glucose, HbA1c, total cholesterol, triglyceride, HDL, low-density lipoprotein (LDL), or serum creatinine in participants with T2D. In the meantime, urinary IL-18 correlates with hemoglobin ($r = -0.218$, $p = 0.026$), albumin ($r = -0.466$, $p < 0.001$), eGFR ($r = -0.393$, $p = 0.001$), and serum IL-18 ($r = 0.448$, $p < 0.001$) in diabetic subjects with DKD. On the other hand, urinary IL-18 correlates with age ($r = 0.209$, $p = 0.025$), male sex ($r = -0.35$, $p < 0.001$), hemoglobin ($r = -0.297$, $p = 0.003$), and serum creatinine ($r = -0.278$, $p = 0.003$) in diabetic subjects without DKD.

We performed univariate analysis of the relationships between the parameters of arterial stiffness and the IL-18 levels in patients with type 2 diabetes (Table 2). The urinary IL-18 levels correlated positively with cf-PWV ($r = 0.309$, $p < 0.001$); however, we found no significant correlation between urinary IL-18 and cr-PWV in participants with T2D. Moreover, we only found significant correlation between the urinary IL-18 levels and cf-PWV ($r = 0.418$, $p < 0.001$) in diabetic subjects with DKD. We found no significant correlation between urinary IL-18 and cf-PWV in diabetic subjects without DKD. Nevertheless, we found no significant correlation between serum IL-18 and cf-PWV or cr-PWV in participants with T2D, diabetic subjects with DKD, or diabetic subjects without DKD.

To further clarify the link between urinary IL-18 and severity of arterial stiffness, we performed multiple regression analysis (Table 3), the association between urinary IL-18 and cf-PWV remained significant when controlling for age and gender (model 1) and additionally controlling for traditional arterial stiffness risk factors (model 2). Moreover, the association was little attenuated when further controlling for UACR and eGFR (model 3). Of note, although serum IL-18 had significant correlations with urinary IL-18 ($r = 0.219$, $p = 0.004$), none of such association was significant when urinary IL-18 and the traditional cardiovascular risk factors were simultaneously included in the model (model 3).

Given the association between urinary IL-18 and cf-PWV, the median cf-PWV was compared across the median of the urinary IL-18 levels. cf-PWV was greater in the higher group of

TABLE 2 Univariate analysis of relationship between logarithmic serum or urinary IL-18 levels and characteristics of type 2 diabetes.

	Total (n = 180)				T2D − DKD (n = 115)				T2D + DKD (n = 65)			
	UIL-18 [a]		SIL-18 [a]		UIL-18 [a]		SIL-18 [a]		UIL-18 [a]		SIL-18 [a]	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	0.224	0.002	0.128	ns.	0.209	0.025	0.088	ns.	0.239	ns.	0.245	ns.
Male	−0.253	0.001	0.020	ns.	−0.350	<0.001	0.056	ns.	−0.170	ns.	−0.010	ns.
Duration of T2D	0.259	<0.001	−0.110	ns.	0.171	ns.	−0.146	ns.	0.216	ns.	−0.086	ns.
BMI	0.044	ns.	0.137	ns.	0.044	ns.	0.285	0.003	−0.124	ns.	−0.065	ns.
Hypertension	0.170	0.023	0.105	ns.	0.128	ns.	0.114	ns.	0.125	ns.	0.119	ns.
SBP	0.228	0.002	0.072	ns.	0.189	0.043	0.019	ns.	0.186	ns.	0.173	ns.
DBP	0.138	ns.	0.055	ns.	0.088	ns.	0.097	ns.	0.059	ns.	0.035	ns.
FBG	0.009	ns.	0.054	ns.	−0.028	ns.	0.200	0.038	0.107	ns.	−0.224	ns.
HbA1c	0.047	ns.	−0.061	ns.	0.011	ns.	0.095	ns.	0.117	ns.	−0.373	0.003
Hb	−0.239	0.002	0.070	ns.	−0.297	0.003	0.071	ns.	−0.218	0.026	0.079	ns.
Albumin	−0.305	<0.001	0.288	<0.001	−0.175	ns.	0.346	<0.001	−0.466	<0.001	0.151	ns.
TC	0.028	ns.	0.157	0.042	0.025	ns.	0.110	ns.	0.049	ns.	0.209	ns.
TG	0.064	ns.	0.179	0.020	0.011	ns.	0.177	ns.	0.059	ns.	0.181	ns.
HDL	−0.093	ns.	−0.070	ns.	−0.072	ns.	−0.074	ns.	−0.026	ns.	−0.120	ns.
LDL	−0.026	ns.	0.190	0.014	−0.038	ns.	0.160	ns.	0.038	ns.	0.219	ns.
Creatinine	−0.037	ns	0.121	ns	−0.278	0.003	0.135	ns.	0.246	0.048	0.206	ns.
eGFR	−0.237	<0.001	−0.172	0.025	−0.094	ns.	−0.162	ns.	−0.393	0.001	−0.255	0.047
UACR ≥ 30 mg/g	0.224	0.003	−0.079	ns.	–	–	–	–	–	–	–	–
UIL-18 [a]	1	–	0.219	0.004	1	–	0.111	ns.	1	–	0.448	<0.001
SIL-18 [a]	0.219	0.004	1	–	0.111	ns.	1	–	0.448	<0.001	1	–
cf-PWV	0.309	<0.001	0.080	ns.	0.164	ns.	−0.004	ns.	0.418	<0.001	0.245	ns.
cr-PWV	−0.044	ns.	−0.072	ns.	−0.002	ns.	−0.118	ns.	−0.192	ns.	0.027	ns.

a, Urinary and serum IL-18 levels were analyzed as naturally logarithmically transformed values.
BMI, body mass index; cr-PWV, carotid–radial pulse wave velocity; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FBG, fast blood glucose; HDL, high-density lipoprotein cholesterol; Hb, hemoglobin; HbA1c, hemoglobin A1c; LDL, low-density lipoprotein cholesterol; SBP, systolic blood pressure; SIL-18, serum interleukin-18; TC, total cholesterol; TG, triglyceride; T2D, type 2 diabetes; UACR, urinary albumin-to-creatinine ratio; UIL-18, urinary interleukin-18. ns, no significance.

urinary IL-18 than in the lower group (Table 4). Moreover, the differences persisted when adjusting traditional atherosclerotic risk factors and the presence of DKD (UACR ≥ 30 mg/g and/or eGFR < 60 ml/min/1.73 m^2).

Discussion

In the present study, we have found in subjects with T2D that the urinary IL-18 levels are associated with increased arterial stiffness as evaluated by cf-PWV. Moreover, the association was independent of the traditional arterial stiffness risk factors and the presence of UACR and eGFR. On the other hand, no significant correlation between serum IL-18 and cf-PWV was found in subjects with T2D. This is the first study that demonstrates the associations between the IL-18 levels and arterial stiffness in patients with T2D, with UACR and eGFR taken into account.

In current study, we found that the urinary IL-18 levels were higher in the group of diabetic subjects with DKD than in the group without DKD. This finding is approximately in line with those studies, suggesting that the higher level of urinary IL-18 has been a promise marker of kidney tubule injury (11, 12, 16). Of note, the positive correlation between the urinary IL-18 levels and cf-PWV was observed only in subjects with DKD, indicating that patients with diabetes with kidney involvement probably have a higher risk factor of CVD, and it was previously reported that subjects with DKD have the highest cardiovascular mortality compared to both patients with T2D without DKD and subjects without diabetes (17). Whether urinary IL-18 is associated with mortality or CVD requires further studies to clarify (18, 19).

The serum IL-18 levels are associated with albuminuria and atherosclerosis in patients with T2D (13). Subjects with elevated serum IL-18 were prone to develop T2D (20). However, elevated serum IL-18 was not associated with a higher risk for primary cardiovascular events in total diabetic population or diabetic subjects with renal dysfunction (21). In accordance with these controversial, we found no significant correlation between serum IL-18 and cf-PWV in participants with T2D, diabetic subjects with DKD, or diabetic subjects without DKD.

We have found that the higher IL-18 levels are associated with greater cf-PWV, suggesting link with arterial stiffness. When controlling for age and gender, urinary IL-18 was significantly associated with cf-PWV, and the association was independent of traditional arterial stiffness risk factors (22). The association remains significant when further controlled for UACR and eGFR, suggesting that the association is independent of kidney function markers. To further demonstrate the associations between IL-18 and arterial stiffness, mean cf-PWV was compared between the higher and lower groups of urinary IL-18. cf-PWV was greater in patients with higher urinary IL-18 than those with lower urinary IL-18, and the difference persisted when adjusting the traditional arterial stiffness risk factors. The differences between IL-18 and cf-PWV were not virtually modified when the presence of DKD was considered, further supporting the link between IL-18 and arterial stiffness.

Urinary IL-18 is produced within the kidney tissue in response to injury and inflammation (12). Urinary IL-18 is also expressed and secreted by macrophages in kidney diseases (23). The correlation between urine molecule markers and arterial stiffness may be explained by the following factors: 1. chronic kidney disease is a well-known risk factor for CVD, which is also a risk factor for

TABLE 3 Multivariable linear regression analysis of relationship between logarithmic urinary IL-18 and cf-PWV.

Variables	Model 1		Model 2		Model 3	
	β	*p*	β	*p*	β	*p*
UIL-18 [a]	0.098	<0.001	0.077	0.005	0.062	0.037
Age	0.007	<0.001	0.006	0.002	0.004	0.028
Male	0.020	0.551	0.072	0.056	0.035	0.415
BMI			0.001	0.837	0.001	0.963
Duration of T2D			0.006	0.017	0.006	0.028
Hypertension			0.130	<0.001	0.098	0.007
Hb			−0.002	0.041	−0.002	0.122
Albumin			0.001	0.843	0.003	0.418
TC			0.027	0.019	0.012	0.388
HDL			0.027	0.649	0.054	0.400
eGFR					−0.001	0.389
UACR ≥ 30 mg/g					0.096	0.012
SIL-18 [a]					0.026	0.499

eGFR, estimated glomerular filtration rate; FBG, fast blood glucose; Hb, hemoglobin; SIL-18, serum interleukin-18; TC, total cholesterol; T2D, type 2 diabetes; UACR, urinary albumin-to-creatinine ratio; UIL-18, urinary interleukin-18.
a, Urinary and serum IL-18 levels were analyzed as naturally logarithmically transformed values.
Model 1: Age and gender. Model 2: Model 1 and traditional arterial stiffness risk factors (BMI, duration of T2D, hypertension, Hb, albumin, TC, and HDL). Model 3: Model 2 and UACR, eGFR, and serum IL-18.

TABLE 4 Median of cf-PWV for subjects with T2D grouped according to the urinary levels of IL-18.

	UIL-18 (pg/mg UCr)		
	≤ 140.82	> 140.82	*p*
cf-PWV (m/s) 95%CI	8.050 (7.634, 8.466)	9.046 (8.629, 9.462)	0.001
cf-PWV (m/s) 95%CI [a]	8.142 (7.719, 8.565)	8.914 (8.561, 9.311)	0.009
cf-PWV (m/s) 95%CI [b]	8.475 (8.036, 8.915)	9.064 (8.679, 9.449)	0.046

cf-PWV, carotid–femoral pulse wave velocity; UCr, urinary creatinine; UIL-18, urinary interleukin-18.
a, Controlling for age and gender.
b, Additionally controlling for traditional arterial stiffness risk factors (BMI, duration of T2D, hypertension, Hb, albumin, TC, and HDL), and the presence of DKD (UACR ≥ 30 mg/g and/or eGFR < 60 ml/min/1.73 m^2).

arterial stiffness; 2. previous studies suggested that changes in markers of kidney injury often reflect renal tissue lesions and impaired renal function, which may affect the cardiovascular system and arterial stiffness through water, acid–base balance, electrolyte and mineral metabolism, endocrine, etc.; 3. urine markers reflect not only kidney disease but also the severity of systemic diseases, including diabetes, which is itself an important cardiovascular risk; 4. urine markers are likely to systematically reflect the extent of systemic lesions, that is, kidney lesions are likely to coexist with other organs in the body, including the heart, blood vessels, liver, and lung. These lesions probably progress together. Further studies may investigate the kidney-related mechanism on the development and progression of arterial stiffness in patients with diabetes and will help understand the reliability of urinary markers as specific biomarkers of CVD.

There are some limitations for the current study. First, we cannot currently determine the causal relationships between the IL-18 levels and greater arterial stiffness because of the cross-sectional design. Second, this study included substantial number of patients with T2D on medications, requiring studies to separate the effects of such medications. Furthermore, the source of urinary IL-18 in patients with T2D is not known. The elevated IL-18 levels in diabetic kidney tissue or infiltrated macrophage in the kidney may be responsible. Further experimental studies are needed to elucidate these questions.

In conclusion, we have demonstrated an association between the higher urinary IL-18 level and greater cf-PWV, suggesting the link between IL-18 and arterial stiffness in patients with T2D. This finding might offer a clue to understand the role of IL-18 in the development of CVDs.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional ethical committee of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Concept the study: JY and YZ; data acquisition: CS, AH, XW, and XZ; data analysis/interpretation: CS, LW, LJ, and YZ; statistical analyses: LJ, JY, and YZ; drafting the work and revising: JY and YZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Diabetic kidney disease progression is associated with decreased lower-limb muscle mass and increased visceral fat area in T2DM patients

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Aim: This study aimed to explore the relationship between lower-limb muscle mass/visceral fat area and diabetic kidney disease (DKD) progression in patients with type 2 diabetes mellitus (T2DM).

Methods: A total of 879 participants with T2DM were divided into 4 groups according to the prognosis of CKD classification from Kidney Disease: Improving Global Outcomes (KDIGO). Rectus femoris cross-sectional area (RFCSA) was measured through ultrasound, and visceral fat area (VFA) was evaluated with bioelectric impedance analysis (BIA).

Results: T2DM patients with high to very high prognostic risk of DKD showed a reduced RFCSA (male $P < 0.001$; female $P < 0.05$), and an enlarged VFA (male $P < 0.05$; female $P < 0.05$). The prognostic risk of DKD was negatively correlated with RFCSA ($P < 0.05$), but positively correlated with VFA ($P < 0.05$). Receiver-operating characteristic analysis revealed that the cutoff points of T2DM duration combined with RFCSA and VFA were as follows: (male: 7 years, 6.60 cm2, and 111 cm2; AUC = 0.82; 95% CI: 0.78–0.88; sensitivity, 78.0%; specificity, 68.6%, P < 0.001) (female: 9 years, 5.05 cm2, and 91 cm2; AUC = 0.73; 95% CI: 0.66–0.81; sensitivity, 73.9%; specificity, 63.3%, P < 0.001).

Conclusion: A significant association was demonstrated between reduced RFCSA/increased VFA and high- to very high-prognostic risk of DKD. T2DM duration, RFCSA, and VFA may be valuable markers of DKD progression in patients with T2DM.

Clinical trial registration: http://www.chictr.org.cn, identifier ChiCTR2100042214.

KEYWORDS

Sarcopenia, abdominal obesity, visceral fat area, diabetic kidney disease, type 2 diabetes mellitus

Introduction

Diabetic kidney disease (DKD) is an important microvascular complication of diabetes, leading to increased mortality in diabetic patients (1). It has been reported that T2DM affects 8.2% of adults (2), 20%–40% of whom are expected to be diagnosed with DKD (3). The only treatment options for late stage DKD include dialysis or kidney transplantation, which are costly, significantly increasing personal and social burdens (4). Hence, identifying and managing the risk factors for DKD is of paramount importance in clinical practice.

Skeletal muscle constituting about 40% of body weight in healthy weight adults falls in quantity and quality with age (5). The mass of skeletal muscle also differs between the sexes. Sarcopenia characterized by gradual skeletal muscle strength and mass deterioration is also considered a complication of DM and has received increasing attention in recent years (6, 7). Many studies have shown that sarcopenia syndrome is commonly found in chronic kidney disease (CKD) patients, mainly those with end-stage kidney disease (ESKD) who received hemodialysis (8). Although previous studies have explored sarcopenia in DM or CKD (9, 10), whether it is associated with DKD is still unclear. No unified definition of sarcopenia has been recommended so far, and the consensus by the European Working Group on Sarcopenia in Older People (EWGSOP) is widely accepted (6, 11). The Asian Working Group for Sarcopenia (AWGS) further provided specific cutoff values for Asian population (12). The assessment of sarcopenia is complex and time-consuming, requiring simple techniques capable of monitoring changes in muscle mass as disease progresses. Douglas W. et al. demonstrated that ultrasound-derived rectus femoris cross-sectional area (RFCSA) appeared to be a reliable index of total quadriceps volume, which was a measure of muscle mass (13). Mueller et al. showed that ultrasound might be a rapid and convenient method to assess sarcopenia (14).

Obesity has become a global health problem due to its associations with coronary artery disease, T2DM, nonalcoholic fatty liver disease, etc. (15, 16). Moreover, some studies have shown that abdominal obesity adversely affects renal prognosis, which is independent of diabetes (17, 18). Previous studies demonstrated that excessive visceral fat area (VFA) was related to insulin resistance and was a crucial risk factor for the development of T2DM compared with waist circumference or body mass index (BMI) (19, 20). The present study was designed to investigate the relationship between RFCSA/VFA and the prognostic risk of DKD, and to elucidate whether RFCSA/VFA was a marker for DKD progression.

Materials and methods

Study design

This controlled, open-label, cross-sectional trial was performed to explore the relationship between RFCSA/VFA and DKD progression. A total of 879 participants were enrolled at the Department of Endocrinology, Shenzhen Hospital, Southern Medical University, China, between March 2020 and December 2021.

Patients included were more than 18 years and were diagnosed with T2DM.

The exclusion criteria were listed as follows: acute complications of diabetes, such as hyperglycemic hyperosmolar coma, hypoglycemic coma, diabetic ketoacidosis and lactic acidosis; nondiabetic nephropathy; myasthenia or muscular atrophy caused by other factors, such as central and peripheral nervous system inflammatory or degenerative diseases, congenital/hereditary diseases, cerebrovascular diseases, craniocerebral trauma, and bone and joint diseases; and malignant tumors, chronic heart failure with decreased ejection fraction, severe liver disease, uncontrolled hypertension, and pregnancy.

The patients' clinical data, such as sex, age, diabetes duration, BMI, blood pressure, history of alcohol consumption, smoking history, were recorded. Laboratory measurements, including blood urea nitrogen (BUN), creatinine (Cr), cystatin C (CysC), serum uric acid (SUA), blood lipid profile, glycosylated hemoglobin (HbA1c), fasting plasma glucose (FPG), fasting C-peptide (FCP), and fasting insulin (FINS), were tested after an 8-h fast. Also, 24-h urinary albumin excretion rate (UAER) and urinary albumin-to-creatinine ratio (UACR) were measured and recorded. Estimated glomerular filtration rate (eGFR) was calculated using CKD-EPI (21, 22). According to the prognosis of CKD classification from Kidney Disease: Improving Global Outcomes (KDIGO) 2020 Clinical Practice Guideline (23), the participants were divided into 4 groups as follows: low risk, moderate risk, high risk and very high-risk groups.

RFCSA assessment using ultrasound

RFCSA was measured by ultrasonography using a 3-12 MHz transducer array (Philips Ultrasound, WA, USA) as previously described (24, 25). All measurements were made by the same sonographer. The patients were asked to keep relaxed, extend legs and to be in a supine position with upper body elevated by 30°. The point 60% of the distance from the anterior superior iliac spine to the superior border of the patella was located, and the ultrasound probe was placed perpendicularly along the superior part of the right thigh to obtain the transverse images of the RF (14).

VFA assessment by BIA

Abdominal VFA was estimated using an Omron DUALSCAN BIA machine (Omron HDS-2000, Kyoto, Japan), which was a multifrequency impedance body composition analyzer. Eight-point tactile electrode method was utilized

following the protocol. Resistance at five specific frequencies (1, 50, 250, 500 kHz, and 1 MHz) and reactance at three specific frequencies (5, 50 and 250 kHz) were measured to obtain the reading of VFA (cm^2) on the screen. All measurements were performed by the same experienced researcher.

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Descriptive data were expressed as mean ± standard deviation for continuous variables with a normal distribution and as median (interquartile range) for non-normal distribution variables. Categorical variables were summarized using percentage or frequency. Continuous data with normal distribution in different groups were compared using independent sample t test or one-way analysis of variance (ANOVA), whereas the Kruskal–Wallis test was performed for parameters with a skewed distribution. Pearson's χ^2 test was employed to analyze categorical data. Spearman's correlation analysis was used to explore the association between different prognostic risks of DKD and clinical characteristics (age, duration, TG, HbA1c, RFCSA, and VFA) of patients with T2DM stratified by sex. Multivariate logistic regression was performed to determine the risk factors for high-/very high-risk prognosis of DKD. Furthermore, receiver-operating characteristic (ROC) analysis was performed to determine the optimal cutoff points of diabetes duration, RFCSA and VFA for indicating high/very high prognostic risk of DKD in male and female patients respectively. All statistical analyses were 2-tailed and a $P < 0.05$ was considered significant.

Results

Baseline characteristics of patients

In total, 941 T2DM patients underwent screening, and 879 participants were enrolled, as 62 were excluded based on exclusion criteria. Of these subjects, 270 patients (30.72%) were diagnosed with DKD according to KDIGO 2020 Clinical Practice Guideline (23). The patients were stratified into 4 groups according to KDIGO prognostic risk classification (low risk, moderate risk, high risk, and very high risk) (23). The baseline characteristics of the participants enrolled are presented in Table 1. Significant differences in sex ($P < 0.05$), age ($P < 0.001$), duration ($P < 0.001$), SBP ($P < 0.001$), DBP ($P < 0.001$), Cr ($P < 0.001$), BUN ($P < 0.001$), CysC ($P < 0.001$), SUA ($P < 0.001$), TG ($P < 0.05$), HDL ($P < 0.05$), HbA1c ($P < 0.05$), FPG ($P < 0.001$), FCP ($P < 0.001$), FINS ($P < 0.05$), UAER ($P < 0.001$), and UACR ($P < 0.001$) were observed among the groups. However, smoking, alcohol consumption, BMI, TC, and LDL displayed nonsignificant differences among the groups. Considering that the muscle content distribution was different between men and women, it was necessary to conduct statistical analysis for each sex. Male or female patients were then divided into two groups: high- to very high-risk and low- to moderate-risk groups. The results showed that RFCSA of the high- to very high-risk group was lower than that of the low- to moderate-risk group (male $P < 0.001$; female $P < 0.05$), whereas VFA of the high- to very high-risk group was higher than that of the low- to moderate-risk group (male $P < 0.05$; female $P < 0.05$) regardless of sex (Table 2).

Correlation analysis between the prognostic risk of DKD and clinical parameters of patients with T2DM

Spearman's correlation was conducted to analyze the relationship between the prognostic risk of DKD and clinical parameters of male and female patients separately, and similar findings were noted. The results showed that the prognostic risk of DKD was negatively correlated with RFCSA (male $r = -0.138$, $P < 0.05$; female $r = -0.194$, $P < 0.05$), and positively correlated with age (male $r = 0.210$, $P < 0.001$; female $r = 0.223$, $P < 0.001$), duration (male $r = 0.291$, $P < 0.001$; female $r = 0.212$, $P < 0.001$), TG (male $r = 0.103$, $P < 0.05$; female $r = 0.124$, $P < 0.05$), and VFA (male $r = 0.139$, $P < 0.05$; female $r = 0.144$, $P < 0.05$). However, no significant association was observed between HbA1c and the prognostic risk of DKD in male or female patients with T2DM (Table 3).

Multivariate logistic regression between the prognostic risk of DKD and clinical variables of patients with T2DM

Age and TG were excluded from multivariate logistic regression due to high inter-correlation between age and duration ($P < 0.001$, data not shown) and between VFA and TG ($P < 0.001$, data not shown). We performed multivariate logistic regression analysis using the prognostic risk of DKD as dependent variable (high-risk and very high-risk group defined as "1", and low-risk and moderate-risk group defined as "0"), and duration, RFCSA and VFA as independent variables. As shown in Table 4, duration (β 1.11, 95% CI 1.07–1.16, $P < 0.001$), RFCSA (β 0.69, 95% CI 0.57–0.83, $P < 0.001$), and VFA (β 1.01, 95% CI 1.00–1.02, $P < 0.05$) was found to be significantly associated with high- to very high-risk prognosis of DKD in male patients with T2DM. Similarly, duration (β 1.04, 95% CI 1.01–1.07, $P < 0.001$), RFCSA (β 0.73, 95% CI 0.59–0.91, $P < 0.05$), and VFA (β 1.01, 95% CI 1.00–1.02, $P < 0.05$) was shown to be significantly linked with high- to very high-risk prognosis of DKD in female T2DM patients (Table 4).

TABLE 1 Clinical characteristics of T2DM patients with different prognosis risk of DKD.

	Low risk(n=609)	Moderately risk (n=174)(n=174)	High risk(n=50)	Very high risk(n=46)	*P*
Sex(M/F)	391/218	120/54	30/20	20/26	<0.05*
Age (years)	53.03 ± 12.02	53.29 ± 13.89	66.50 ± 9.44	61.72 ± 12.07	<0.001**
Duration (years)	5.0 (1.0, 10.0)	8.0 (1.0, 13.0)	10.0 (7.0, 17.0)	16.0 (8.0, 20.0)	<0.001**
BMI (kg/m2)	20.60 ± 4.77	21.55 ± 6.03	21.26 ± 6.53	19.52 ± 7.08	>0.05
SBP (mmHg)	126.60 ± 15.46	134.55 ± 19.32	136.42 ± 18.92	139.00 ± 19.65	<0.001**
DBP (mmHg)	78.04 ± 9.76	81.94 ± 12.99	78.18 ± 11.49	78.20 ± 11.36	<0.001**
Alcohol (%)	17.7%	18.4%	8.0%	10.9%	>0.05
Smoking (%)	27.8%	25.3%	16.0%	15.2%	>0.05
BUN (mmol/L)	4.78 ± 1.37	5.17 ± 1.69	7.35 ± 2.45	9.39 ± 3.66	<0.001**
Cr (μmol/L)	68.31 ± 16.18	77.25 ± 23.96	108.10 ± 26.40	153.33 ± 79.08	<0.001**
CysC (mg/mL)	0.91 ± 0.16	1.02 ± 0.24	1.74 ± 0.98	2.90 ± 2.44	<0.001**
SUA (μmol/L)	325.65 ± 90.39	374.12 ± 113.41	363.24 ± 103.55	362.11 ± 103.68	<0.001**
TG (mmol/L)	1.53(1.04, 2.30)	1.82(1.24,3.18)	1.67 (0.96, 2.87)	1.76(1.42, 2.88)	<0.001**
TC (mmol/L)	4.43 ± 1.44	4.61 ± 1.85	4.16 ± 1.20	4.47 ± 1.37	>0.05
LDL (mmol/L)	2.81 ± 1.08	3.59 ± 1.26	2.62 ± 1.08	2.61 ± 1.10	>0.05
HDL (mmol/L)	1.20 ± 0.34	1.08 ± 0.34	1.14 ± 0.36	1.16 ± 0.25	<0.05*
HbA1C (%)	9.28 ± 2.49	9.74 ± 2.38	9.01 ± 2.66	8.77 ± 2.14	<0.05*
FPG (mmol/L)	8.09 ± 2.94	9.09 ± 3.14	7.75 ± 3.30	7.61 ± 3.06	<0.001**
FCP (ng/mL)	2.03 ± 1.27	2.24 ± 1.20	2.78 ± 1.73	2.80 ± 1.57	<0.001**
FINS (μU/mL)	6.75(4.07, 11.53)	8.13(4.96, 13.05)	9.39(3.87, 16.94)	6.82(4.97, 11.61)	<0.05*
UAER (mg/24h)	7.80 (4.84, 14.62)	55.71 (36.48, 140.15)	66.08 (18.67, 196.64)	90.96 (43.97, 1502.05)	<0.001**
UACR (mg/mmoL)	0.68 (0.41, 1.40)	4.16 (2.09, 15.19)	6.09 (2.36, 32.31)	12.50 (4.15, 171.43)	<0.001**

Values were expressed as mean ± SD for normally distributed data and median with interquartile range for non-normally distributed data, or n (%). Differences among the groups were analyzed by ANOVA for normally distributed values and by the Kruskal-Wallis test for nonparametric values. Pearson's $\chi 2$ test was employed to analyze categorical data. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; BUN, blood urea nitrogen; Cr, creatinine; CysC, Cystatin C; SUA, serum uric acid; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA1c, glycated hemoglobin; FPG, fasting plasma glucose; FCP, fasting C-peptide; FINS, fasting insulin; UAER, urinary albumin excretion rate; UACR, urinary albumin to creatinine ratio. *P < 0.05. **P < 0.001.

ROC analysis

We performed ROC analysis to investigate the optimal cutoff points for diabetes duration, RFCSA and VFA, which could be used to distinguish a high- to very high-risk prognosis of DKD. Multivariate logistic regression analysis was carried out to assess the predictive capability of the combined parameters of diabetes duration, RFCSA and VFA, which were used as independent variables for multivariable ROC analysis. For T2DM male patients, the cutoff values of diabetes duration, RFCSA and VFA were revealed as 7 years, 6.60 cm^2 and 111 cm^2, respectively, with an AUC of 0.82 (95% CI: 0.78–0.88), a sensitivity of 78.0%, and a specificity of 68.6% ($P < 0.001$) (Figure 1 blue). For T2DM female patients, the cutoff of diabetes duration, RFCSA and VFA were 9 years, 5.05 cm^2 and 91 cm^2, respectively, the AUC was 0.73 (95% CI: 0.66–0.81), the sensitivity was 73.9%, and the specificity was 63.3% ($P < 0.001$) (Figure 1 green).

TABLE 2 RFCSA and VFA of T2DM patients with different prognosis risk of DKD.

	Low-Moderately risk	High-Very high risk	*P*
N (Male)	511	50	
RFCSA (cm^2)	7.59 ± 2.61	6.21 ± 1.78	<0.001**
VFA (cm^2)	103.1 ± 45.6	116.2 ± 33.4	<0.05*
N (Female)	272	46	
RFCSA (cm^2)	5.58 ± 1.92	4.64 ± 1.44	<0.05*
VFA (cm^2) F	86.4 ± 36.7	99.4 ± 40.0	<0.05*

Values were expressed as mean ± SD for normally distributed data. Differences between the groups were analyzed by student's t-test for normally distributed values. RFCSA, rectus femoris cross-sectional area; VFA, visceral fat area. *P < 0.05. **P < 0.001.

TABLE 3 Spearman's correlation analysis of different prognosis risk of DKD with Clinical characteristics in T2DM patients stratified by gender.

		r	*P*
Male	**Age**	0.210	<0.001**
	Duration	0.291	<0.001**
	TG	0.103	<0.05*
	HbA1C	-0.060	>0.05
	RFCSA	-0.138	<0.05*
	VFA	0.139	<0.05*
Female	**Age**	0.223	<0.001**
	Duration	0.212	<0.001**
	TG	0.124	<0.05*
	HbA1C	0.039	>0.05
	RFCSA	-0.194	<0.05*
	VFA	0.144	<0.05*

TG, triglycerides; HbA1c, glycated hemoglobin; RFCSA, rectus femoris cross-sectional area; VFA, visceral fat area. *P < 0.05. **P < 0.001.

Discussion

DKD is a major cause of CKD worldwide and brings enormous economic burden to patients and society (26). In addition to the use of medication to control hyperglycemia and hypertension, modifying other related factors is of great importance for the management of DKD patients. Sarcopenia is a frequent condition reported in CKD patients and is considered to be linked with an increased risk of hospitalization and all-cause mortality (27). Previous studies have shown that sarcopenia reflects progressive and cumulative effects of CKD on skeletal muscle (13, 28). Abdominal obesity is a risk factor for multiple complications of diabetes. Heng Wan et al. showed that abdominal obesity was strongly associated with DKD (29).

In the present study, the patients were divided into two groups (high- to very high-risk group and low- to moderate-risk group) to explore the relationship between RFCSA/VFA and the prognostic risk of DKD. The results showed an obviously reduced RFCSA in the high- to very high-risk group compared with the low- to moderate-risk group. Although sarcopenia has been extensively explored in patients with diabetes or CKD (9, 30, 31), the changes of RFCSA in DKD patients has not yet been reported. Many studies have shown that the incidence rate of sarcopenia in ESKD patients is higher than that in patients with early-stage renal disease, which is consistent with our results (8). Some studies reported that abdominal obesity, compared with general obesity, had a greater impact on the risk of DKD (32, 33). Chin-Hsiao Tseng demonstrated a close and independent association between abdominal obesity and elevated UAER in female patients with diabetes but not in male diabetic patients (34). Our results showed an enlarged VFA in high- to very high-risk male and female DKD patients.

Furthermore, our study showed that the prognostic risk of DKD was positively correlated with age, duration and TG, which were recognized as important factors influencing the progression of DKD. A systematic review and meta-analysis of 20 cohorts comprising 41,271 individuals showed that the independent risk factors for DKD development were duration, age, smoking status, HbA1c, TG, HDL-C, BMI, SBP, UACR, and eGFR (35). In the present study, no relationship was established between HbA1c and DKD, which was different from the conclusions of previous studies (36). This discrepancy could be explained by the fact that HbA1c only reflected glycemic control in the recent 3 months. In addition, another explanation might be that some DKD patients were complicated with renal anemia, resulting in lower HbA1c concentration compared with the actual level.

This study also showed that the prognostic risk of DKD was negatively correlated with RFCSA. The exact underlying mechanism has not been fully elucidated. However, abnormal renal function and hyperglycemia are considered essential factors for sarcopenia in patients with DKD. Firstly, sarcopenic obesity, a combination of sarcopenia and obesity, reflects a vicious link between insulin resistance and sarcopenia. Obesity-induced insulin resistance triggers a series of events that lead to a decrease in muscle glucose supply and quantitative and qualitative deterioration of muscles, further enhancing insulin resistance and creating a vicious cycle (37). Secondly, accumulation of advanced glycation end-products (AGEs) and diabetic vasculopathy may also impair muscle mass and

TABLE 4 Risk factors for high-/very high-risk prognosis of DKD in multivariate logistic regression.

	Independent variables	β (95% Cl)	*P*
Male	**Duration**	1.11(1.07, 1.16)	<0.001**
	RFCSA	0.69 (0.57, 0.83)	<0.001**
	VFA	1.01 (1.00, 1.02)	<0.05*
Female	**Duration**	1.04(1.01, 1.07)	<0.05*
	RFCSA	0.73 (0.59, 0.91)	<0.05*
	VFA	1.01 (1.00, 1.02)	<0.05*

RFCSA, rectus femoris cross-sectional area; VFA, visceral fat area. *P < 0.05. **P < 0.001.

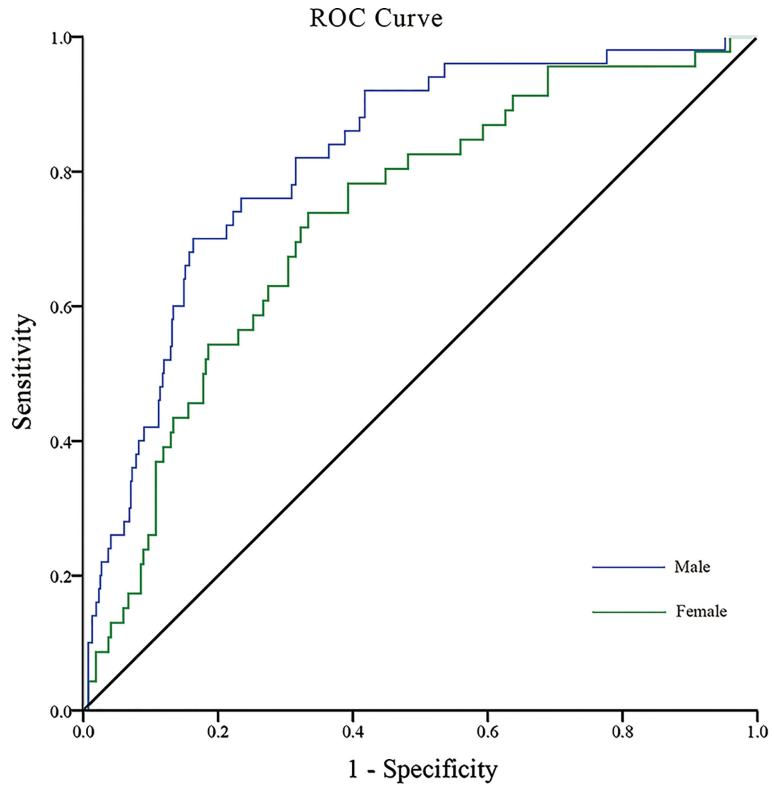

FIGURE 1
ROC analysis of T2DM duration combined with RFCSA and VFA to predict high-/very high-risk prognosis of DKD in male/female T2DM patients. [Male (blue): AUC=0.82; 95% CI: 0.78–0.88; Sensitivity 78.0%, Specificity 68.6%; *P* < 0.001] [Female (green): AUC=0.73; 95% CI: 0.66–0.81; Sensitivity 73.9%, Specificity 63.3%, P < 0.001].

strength, leading to sarcopenia (38–40). Thirdly, with the worsening of renal function, sarcopenia occurs due to accelerated protein catabolism, and reduced energy and protein intake during dialysis (8).

The relationship between DKD and abdominal obesity was investigated in many previous studies. A meta-analysis, including 2205 patients with VFA measurements from 3 cross-sectional studies, demonstrated that VFA was associated with greater odds of DKD in patients with type 2 diabetes (34). Asakawa H et al. showed that VFA level was significantly higher in patients with DKD than those without DKD (41). However, some studies showed the contradictory conclusions. Man et al. (42) found that abdominal obesity had no association with DKD in patients with T2DM. Therefore, the relationship between abdominal obesity and DKD deserves further investigation. The present study found that VFA was positively correlated with the prognostic risk of DKD. Although the mechanisms underlying the linking between DKD and abdominal obesity are still unclear, several hypotheses may be proposed. Firstly, excessive visceral fat accumulation leads to systemic inflammation, which may contribute to a cascade of events such as insulin resistance, oxidative stress, and renal damage (43, 44). Secondly, the renin–angiotensin system (RAS) is activated by adipose tissue, which changes sodium retention and renal hemodynamics, ultimately leading to renal damage (45, 46). Thirdly, other metabolic syndromes that are associated with obesity also play an important role in the occurrence and development of DKD (47, 48).

Based on the results of this study, we suggested that the loss of lower-limb muscle mass and the increase in VFA were closely related to the progression of DKD. The prognostic risk of DKD was high or very high for male T2DM patients, with a duration of more than 7 years, a RFCSA of less than 6.60 cm^2, and a VFA of more than 111 cm^2. The prognostic risk of DKD was also high or very high for female T2DM patients, with a duration being more than 9 years, a RFCSA being less than 5.05 cm^2, and a VFA being more than 91 cm^2. Therefore, we speculated that the modified lifestyle to increase skeletal muscle mass and reduce visceral fat accumulation might delay the progression of DKD in patients with T2DM.

This study had some limitations. Firstly, certain confounding factors, such as the level of physical activity and

the use of anti-diabetes medication, might also influence the results of the study. Secondly, the current conclusion was summarized from a cross-sectional trial. Thirdly, VFA was measured using a novel BIA device that has yet only received limited validation (49) rather than a more accurate and reliable method such as computed tomography (CT).

Conclusions

The lower-limb muscle mass of T2DM patients decreased whereas VFA increased with the progression of DKD. The prognostic risk of DKD was negatively correlated with RFCSA but positively correlated with VFA. T2DM duration, RFCSA and VFA were found to be markers of DKD progression. Based on the conclusion of this study, for patients who have not developed DKD or are in the early stage of DKD, individualized lifestyle guidance (including diet and exercise) and reasonable hypoglycemic medicine selection should be given to increase muscle content and reduce abdominal fat, which may delay the occurrence and progress of DKD. In the future, cohort study and fundamental research are needed to verify the viewpoint and further explore relevant mechanisms.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Medical ethics committee of Shenzhen Hospital, Southern Medical University (Approval No. NYSZYYEC20200035). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Each author has made an important scientific contribution to the study and is thoroughly familiar with the primary data. XL and ZC carried out the clinical studies, participated in the statistical analysis and drafted the manuscript. HH and JZ carried out the data acquisition, participated in the manuscript preparation and literature research. LX conceived of the study, and participated in its design and helped to review the manuscript. All authors listed have read the complete manuscript and have approved submission of the paper.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Development and internal validation of a risk model for hyperuricemia in diabetic kidney disease patients

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Purpose: This research aimed to identify independent risk factors for hyperuricemia (HUA) in diabetic kidney disease (DKD) patients and develop an HUA risk model based on a retrospective study in Ningbo, China.

Patients and methods: Six hundred and ten DKD patients attending the two hospitals between January 2019 and December 2020 were enrolled in this research and randomized to the training and validation cohorts based on the corresponding ratio (7:3). Independent risk factors associated with HUA were identified by multivariable logistic regression analysis. The characteristic variables of the HUA risk prediction model were screened out by the least absolute shrinkage and selection operator (LASSO) combined with 10-fold cross-validation, and the model was presented by nomogram. The C-index and receiver operating characteristic (ROC) curve, calibration curve and Hosmer–Lemeshow test, and decision curve analysis (DCA) were performed to evaluate the discriminatory power, degree of fitting, and clinical applicability of the risk model.

Results: Body mass index (BMI), HbA1c, estimated glomerular filtration rate (eGFR), and hyperlipidemia were identified as independent risk factors for HUA in the DKD population. The characteristic variables (gender, family history of T2DM, drinking history, BMI, and hyperlipidemia) were screened out by LASSO combined with 10-fold cross-validation and included as predictors in the HUA risk prediction model. In the training cohort, the HUA risk model showed good discriminatory power with a C-index of 0.761 (95% CI: 0.712–0.810) and excellent degree of fit (Hosmer–Lemeshow test, $P > 0.05$), and the results of the DCA showed that the prediction model could be beneficial for patients when the threshold probability was 9–79%. Meanwhile, the risk model was also well validated in the validation cohort, where the C-index was 0.843 (95% CI: 0.780–0.906), the degree of fit was good, and the DCA risk threshold probability was 7–100%.

Conclusion: The development of risk models contributes to the early identification and prevention of HUA in the DKD population, which is vital for preventing and reducing adverse prognostic events in DKD.

KEYWORDS

diabetic kidney disease, hyperuricemia, independent risk factors, prediction model, nomogram

Introduction

Diabetes mellitus (DM) is a metabolic disease caused by a combination of genetic, environmental factors and dietary habits and is characterized by chronic elevation of blood glucose and inadequate insulin secretion. With the prolonged course of DM and poor long-term glycemic control, the accumulation of abnormal substances in the metabolic process (such as advanced glycosylation end products, free fatty acids, and inflammation-related mediators) can cause functional damage to multiple organs of the body, including the kidneys, retina, and heart and brain vessels. Among them, diabetic kidney disease (DKD) and cardiovascular disease are the leading causes of death and disability in diabetic patients, posing a significant threat to human physical and mental health.

DKD is one of the most important microvascular complications of DM, with an increased urinary albumin excretion rate and reduced glomerular filtration rate as the main clinical manifestations (1). The main pathological changes of DKD are proliferation of thylakoid cells, extracellular matrix accumulation, basement membrane thickening, diffuse glomerulosclerosis, and interstitial fibrosis (2, 3). In recent years, as the prevalence of DM has increased globally, the prevalence of DKD has also increased, with approximately 40% of DM patients suffering from DKD, which is a significant cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) (4). Uric acid (UA) is the end product of the metabolism of purine compounds with a dynamic balance of production and clearance under normal conditions. However, the disruption of the balance inevitably causes a continuous increase in UA levels, which in turn results in the development of hyperuricemia (HUA). The kidney plays an important role in the excretion of uric acid, of which approximately 90% of HUA is the result of abnormal glomerular and/or tubular function (5).

The public has increasingly recognized HUA as a risk factor for DKD (6–8), and UA may become a new therapeutic target for DKD. However, other studies have shown no causal relationship between elevated UA levels and kidney disease only as a downstream marker of kidney damage (9, 10). Few studies on risk factors for HUA in the DKD population have been reported. There have been many studies on HUA risk prediction models, but most were developed based on healthy populations. Cao et al. (11) developed a simple HUA Cox proportional hazard model based on an urban Chinese population that showed good clinical discrimination between men and women [C-index: 0.783 (95% CI: 0.779–0.786) vs. 0.784 (95% CI: 0.778–0.789)]. Gao et al. (12) constructed a random forest prediction model for health checkups. In addition, risk prediction models based on machine learning, such as artificial neural networks, are also used for HUA prediction (13, 14). The predictive model is established to serve the clinic better, so the characteristics of solid predictive ability, visualization, and easy operation are necessary. The least absolute shrinkage and selection operator (LASSO) combined with 10-fold cross-validation was used to screen for characteristic variables, while the nomogram provides a tool for the visual representation of predictive models. The establishment of HUA risk prediction would contribute to the early intervention of DKD, the delay of the disease course, and the reduction of adverse prognostic events. The purpose of this study was, on the one hand, to identify independent risk factors for HUA in the DKD population and, on the other hand, to develop a risk model for HUA with the help of the nomogram.

Materials and methods

Patients

From January 2019 to December 2020, questionnaires were administered to T2DM patients who were outpatients and inpatients in two hospitals in Ningbo, including the Affiliated Hospital of Medical School, Ningbo University, Yinzhou No. 3 Hospital. Relevant clinical data were obtained and recorded through questionnaires, physical examinations, and laboratory tests. To ensure the accuracy of the study, the completeness of each individual data was checked, those with more missing values (exceeding 20% of the total) were removed, and those with fewer missing values (<20% of the total) were filled with multiple imputation (15). After data processing, complete information was obtained for 1,682 T2DM patients. Finally, 610 patients with clearly diagnosed DKD were included in the study by reviewing past medical history and inquiry. The diagnosis of DKD meets one of the following criteria (16): (1) random urine albumin creatinine ratio (ACR) ≥30 mg/g or urinary albumin excretion rate ≥30 mg/24 h, and the critical value is reached or exceeded in two out of three tests within 3 to 6 months; (2) estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2 for more than 3 months; (3) renal biopsy consistent with DKD pathological changes. The study was approved by the ethics committee of the Affiliated Hospital of Medical School, Ningbo University (KY20171112), and written informed consent was obtained from all participants.

Inclusion criteria: T2DM; age ≥18 years; clearly diagnosed DKD. exclusion criteria: other renal diseases; severe life-threatening organ dysfunction of the heart, lungs, kidney and liver; tumors; hormone use within the past 6 months.

Procedure

The demographic and clinical data for this study were primarily information that was readily available, relatively complete, and comparable in clinical practice, which was collected through a questionnaire. All staffs involved in the questionnaire received standardized training. The questionnaire

survey collected participants' general characteristics [gender, age, family history of DM, duration of T2DM, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP)], biochemical indicators in the last 3 months [glycated hemoglobin A1c (HbA1c), fasting blood glucose (FBG), postprandial (2 h) blood glucose (PBG), triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and UA], and chronic complications. Biochemical indicators were obtained from each hospital's electronic laboratory record system. Confirmation of chronic complications required a review of medical history and inquiry and was recorded only if there was a clear previous diagnosis.

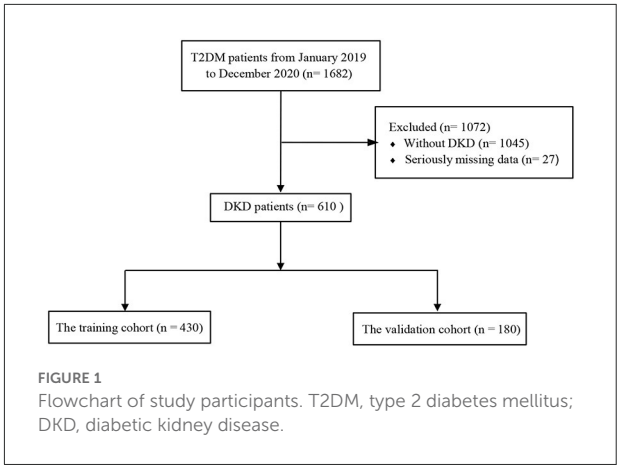

FIGURE 1
Flowchart of study participants. T2DM, type 2 diabetes mellitus; DKD, diabetic kidney disease.

Statistical analysis

Six-hundred ten patients with DKD were enrolled in this research, and data information for all variables was expressed as counts (%). Statistical analysis was performed with R software (version 4.1.2; https://www.R-project.org). Comparison of the count data between the two groups was performed by chi-square test. All tests were two-tailed, and a P value of <0.05 was considered statistically significant.

Participants were randomized to training and validation cohorts according to a certain ratio (7:3) (17, 18), while the random sampling process used the createDataPartition function in the caret package. In addition, we knew from the calculation that the sample size was sufficient for the subsequent statistical analysis, which complied with the rule of 10 events per variable (19, 20). Independent risk factors were identified by multivariable logistic regression analysis. The LASSO is a method applied for data dimensional reduction (21, 22), which could construct a penalty function to obtain a double-standard error. The characteristic variables associated with DKD were screened out by LASSO combined with 10-fold cross-validation. Finally, the HUA risk prediction model is constructed by logistic regression analysis and presented by nomogram (23). The participant screening flow diagram for this study is shown in Figure 1.

The risk predictive models were evaluated in terms of discriminatory ability [C-index and receiver operating characteristic (ROC) curve], calibration ability (Hosmer–Lemeshow test and calibration curve), and clinical applicability [decision curve analysis (DCA)] (17).

Results

Characteristics of the research cohort

Six hundred and ten participants were enrolled in this study, including 412 individuals with DKD without HUA and 198 individuals with DKD with HUA. The percentage of HUA in the DKD population was found to be as high as 32.4% in the study. We observed a similar proportion of males in both groups (52.4 vs. 53.5%), an overwhelming majority of age >60 years (73.5 vs. 75.8%), and a predominance of T2DM duration of 15–20 years (34.7 vs. 26.3%). In the DKD population, the HUA group had a higher proportion of smoking history, drinking history, obese patients, FBG >7 mmol/L, HbA1c >8%, hypertension and eGFR $\leq$ 120 mL/min/1.73m^2, and hyperlipidemia compared with the control group. BMI ($P = 0.025$), SBP ($P = 0.004$), PBG ($P = 0.017$), HbA1c ($P < 0.001$), UA ($P < 0.001$), eGFR ($P < 0.001$) and hypertension ($P < 0.001$) were found to be significantly different between the two groups by univariate analysis (Table 1). A total of 430 (135 with HUA) and 180 (63 with HUA) participants were assigned to the training and validation cohorts, respectively, by randomization sampling, while it could be seen that the variables did not differ in the training and validation cohorts (Table 1).

Independent risk factors

These variables were incorporated into multivariate logistic regression analyses according to the results of the univariate analysis in Table 1 (with a screening criterion of $P < 0.1$). BMI, HbA1c, eGFR, and hyperlipidemia were identified as independent risk factors for HUA in the DKD population (Table 2).

Construction of predictive models

In the training cohort, seven nonzero characteristic variables, such as gender, family history of T2DM, drinking history, BMI, UA, eGFR, and hyperlipidemia, were screened out by LASSO combined with 10-fold cross-validation (Figure 2; Table 3). Since UA is one of the diagnostic criteria for HUA, we selected gender, family history of T2DM, drinking history, BMI,

TABLE 1 Characteristics of participants in different cohorts.

	DKD without HUA	DKD with HUA	*P*-Value	Training cohort (135 with HUA)	Validation cohort (63 with HUA)	*P*-Value
N	412	198		430	180	
gender (male), %	216 (52.4)	106 (53.5)	0.863	234 (54.4)	88 (48.9)	0.215
Age, %			0.595			0.859
≤50 years old	27 (6.6)	15 (7.6)		29 (6.7)	13 (7.2)	
50–60 years old	82 (19.9)	33 (16.7)		79 (18.4)	36 (20.0)	
> 60 years old	303 (73.5)	150 (75.8)		322 (74.9)	131 (72.8)	
Education level, %			0.712			0.146
Primary/illiterate	283 (68.7)	135 (68.2)		286 (66.5)	132 (73.3)	
Middle/high school	121 (29.4)	61 (30.8)		138 (32.1)	44 (24.4)	
University and above	8 (1.9)	2 (1.0)		6 (1.4)	4 (2.2)	
Family history of T2DM, %	86 (20.9)	41 (20.7)	0.999	89 (20.7)	38 (21.1)	0.913
Smoking history, %	92 (22.3)	52 (26.3)	0.309	106 (24.7)	38 (21.1)	0.403
Drinking history, %	67 (16.3)	38 (19.2)	0.363	76 (17.7)	29 (16.1)	0.724
Duration of T2DM, %			0.070			0.121
≤5 years	8 (1.9)	5 (2.5)		9 (2.1)	4 (2.2)	
5–10 years	138 (33.5)	60 (30.3)		131 (30.5)	67 (37.2)	
10–15 years	73 (17.7)	48 (24.2)		88 (20.5)	33 (18.3)	
15–20 years	143 (34.7)	52 (26.3)		149 (34.7)	46 (25.6)	
> 20 years	50 (12.1)	33 (16.7)		53 (12.3)	30 (16.7)	
BMI, %			0.025			0.353
≤24 kg/m^2	184 (44.7)	69 (34.8)		175 (40.7)	78 (43.3)	
24–28 kg/m^2	171 (41.5)	88 (44.4)		190 (44.2)	69 (38.3)	
> 28 kg/m^2	57 (13.8)	41 (20.7)		65 (15.1)	33 (18.3)	
SBP, %			0.004			0.677
≤140 mmHg	269 (65.3)	101 (51.0)		256 (59.5)	114 (63.3)	
140–160 mmHg	98 (23.8)	73 (36.9)		126 (29.3)	45 (25.0)	
160–180 mmHg	40 (9.7)	21 (10.6)		43 (10.0)	18 (10.0)	
> 180 mmHg	5 (1.2)	3 (1.5)		5 (1.2)	3 (1.7)	
DBP, %			0.562			0.428
≤90 mmHg	388 (94.2)	183 (92.4)		398 (92.6)	173 (96.1)	
90–100 mmHg	16 (3.9)	12 (6.1)		23 (5.3)	5 (2.8)	
100–110 mmHg	6 (1.5)	3 (1.5)		7 (1.6)	2 (1.1)	
>110 mmHg	2 (0.5)	0 (0.0)		2 (0.5)	0 (0.0)	
FBG, %			0.633			0.754
≤7 mmol/L	174 (42.2)	91 (46.0)		191 (44.4)	74 (41.1)	
7–11 mmol/L	209 (50.7)	96 (48.5)		211 (49.1)	94 (52.2)	
>11 mmol/L	29 (7.0)	11 (5.6)		28 (6.5)	12 (6.7)	
PBG, %			0.017			0.628
≤11 mmol/L	128 (31.1)	85 (42.9)		147 (34.2)	66 (36.7)	
11–15 mmol/L	185 (44.9)	75 (37.9)		182 (42.3)	78 (43.3)	
>15 mmol/L	99 (24.0)	38 (19.2)		101 (23.5)	36 (20.0)	
HbA1c, %			<0.001			0.372
≤8%	152 (36.9)	110 (55.6)		179 (41.6)	83 (46.1)	
8–10%	143 (34.7)	58 (29.3)		149 (34.7)	52 (28.9)	
>10%	117 (28.4)	30 (15.2)		102 (23.7)	45 (25.0)	
TC (>6.2 mmol/L), %	37 (9.0)	16 (8.1)	0.761	35 (8.1)	18 (10.0)	0.528
TG (>4.1 mmol/L), %	71 (17.2)	43 (21.7)	0.185	81 (18.8)	33 (18.3)	0.910
LDL (>3.4 mmol/L), %	68 (16.5)	22 (11.1)	0.088	66 (15.3)	24 (13.3)	0.617

(Continued)

TABLE 1 (Continued)

	DKD without HUA	DKD with HUA	*P*-Value	Training cohort (135 with HUA)	Validation cohort (63 with HUA)	*P*-Value
HDL (>1 mmol/L), %	265 (64.3)	114 (57.6)	0.110	271 (63.0)	108 (60.0)	0.522
UA, %			<0.001			0.890
≤ 360 μmol/L	325 (78.9)	17 (8.6)		245 (57.0)	97 (53.9)	
360–420 μmol/L	73 (17.7)	37 (18.7)		76 (17.7)	34 (18.9)	
420–480 μmol/L	10 (2.4)	49 (24.7)		40 (9.3)	19 (10.6)	
>480 μmol/L	4 (1.0)	95 (48.0)		69 (16.0)	30 (16.7)	
eGFR, %			<0.001			0.164
>120, mL/min/1.73 m^2	320 (77.7)	70 (35.4)		269 (62.6)	121 (67.2)	
90–120, mL/min/1.73 m^2	55 (13.3)	39 (19.7)		76 (17.7)	18 (10.0)	
60–90, mL/min/1.73 m2	25 (6.1)	46 (23.2)		49 (11.4)	22 (12.2)	
30–60, mL/min/1.73 m^2	7 (1.7)	34 (17.2)		27 (6.3)	14 (7.8)	
≤30, mL/min/1.73 m^2	5 (1.2)	9 (4.5)		9 (2.1)	5 (2.8)	
Hypertensive, %	310 (75.2)	179 (90.4)	<0.001	356 (82.8)	133 (73.9)	0.014
Hyperlipidemia, %	204 (49.5)	115 (58.1)	0.057	227 (52.8)	92 (51.1)	0.723
Atherosclerosis, %	394 (95.6)	187 (94.4)	0.545	405 (94.2)	176 (97.8)	0.062
CVD, %	103 (25.0)	54 (27.3)	0.554	109 (25.3)	48 (26.7)	0.761

DKD, diabetic kidney disease; HUA, hyperuricemia; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; PBG, postprandial (2 h) blood glucose; TC, total cholesterol; TG, triglycerides; LDL, low–density lipoprotein; HDL, high–density lipoprotein; UA, uric acid; eGFR, estimated glomerular filtration rate; CVD, cardiovascular disease.

TABLE 2 Multivariate logistic regression analysis.

Variable	Coefficients	Odds Ratio (95% CI)	*P*-value
BMI			
≤24 kg/m^2		1	1
24–28 kg/m^2	0.441	1.554 (1.002–2.426)	<0.05
>28 kg/m^2	0.83	2.294 (1.310–4.021)	<0.01
HbA1c			
≤8%		1	1
8–10%	−0.406	0.666 (0.424–1.041)	>0.05
>10%	−0.707	0.493 (0.287–0.834)	<0.01
eGFR			
>120 mL/min/1.73 m^2		1	1
90–120 mL/min/1.73 m^2	1.200	3.322 (1.996–5.528)	<0.01
60–90 mL/min/1.73 m^2	2.144	8.541 (4.859–15.367)	<0.01
30–60 mL/min/1.73 m^2	3.211	24.814 (10.849–64.722)	<0.01
≤30 mL/min/1.73 m^2	2.221	9.214 (2.892–32.562)	<0.01
Hyperlipidemia	0.600	1.822 (1.216–2.758)	<0.01

BMI, body mass index; eGFR, estimated glomerular filtration rate.

eGFR, and hyperlipidemia as predictors to construct the HUA risk model by logistic regression analysis, which was visualized by nomogram (Figure 3).

Validation of predictive models

The C-index and the area under the ROC curve (AUC) were used to assess the discriminatory ability of the risk model. In the training cohort, the C-index was 0.761 (95% CI: 0.712–0.810), and the AUC was 0.761, while in the validation cohort, the values were 0.843 (95% CI: 0.780–0.906) and 0.843 (Figure 4).

From the calibration curve, the predicted values were very close to the theoretical values in the training and validation cohorts, showing an excellent degree of fit (Figure 5), which was further confirmed by the Hosmer–Lemeshow test ($P > 0.05$) (Table 4).

DCA is a method that has been used to evaluate the clinical applicability of risk models. Figure 6 shows that the risk threshold probabilities for the training and validation cohorts were 9–79% and 7–100%, respectively, which suggested that the risk prediction model could benefit patients within this threshold probability range.

Discussion

DKD seriously affects the quality of life of T2DM patients and threatens their lives, while an increasing number of scholars have started to pay attention to and study the relationship between UA and DKD (24–26). Through a retrospective investigation in Ningbo, China, 610 DKD patients were enrolled, including 198 HUA patients. The multivariate logistic regression analysis identified BMI, HbA1c, eGFR, and hyperlipidemia as

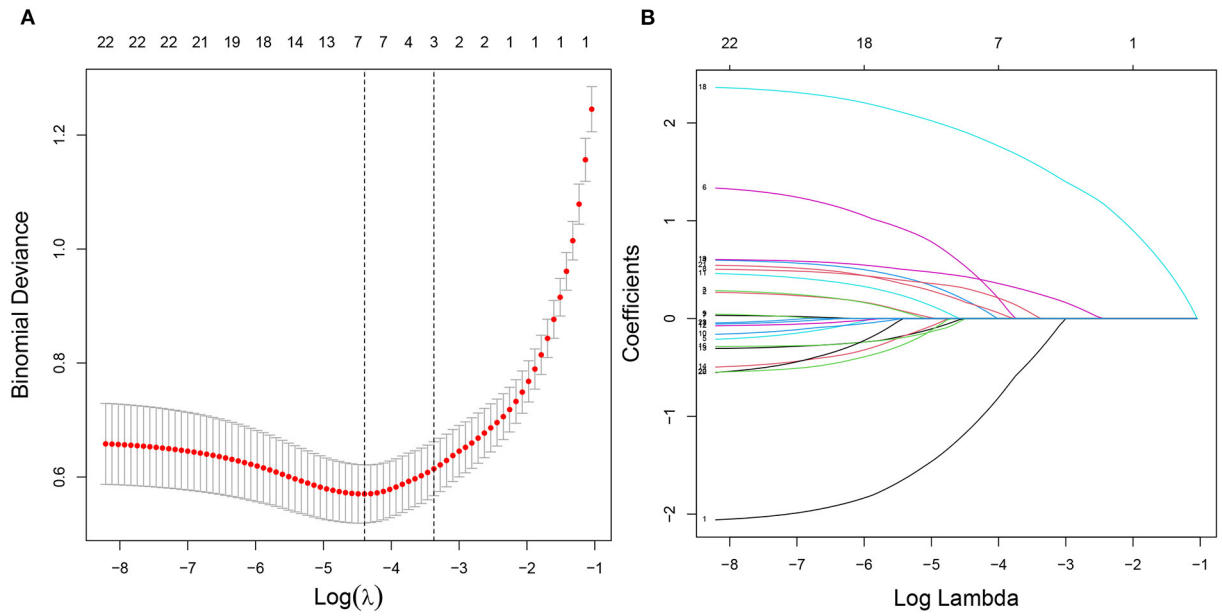

FIGURE 2
Characteristic variables were screened using LASSO regression analysis. **(A)** The selection of the best parameter (lambda) in the LASSO model uses 10-fold cross-validation with the lowest standard. The relationship curve between partial likelihood deviation (binomial deviation) and log (lambda) was plotted. Dotted vertical lines were drawn at the optimal values by using the minimum criteria and the one SE of the minimum criteria (the one SE criteria). **(B)** LASSO coefficient profiles of the seven characteristic variables. A coefficient profile plot was produced against the log (lambda) sequence. LASSO, least absolute shrinkage and selection operator; SE, standard error.

TABLE 3 Coefficients and lambda.min value of the LASSO regression.

Variables	Coefficients	Lambda.min
Gender	−1.106	0.012
Family history of T2DM	0.154	
Drinking history	0.475	
BMI	0.291	
UA	1.879	
eGFR	0.416	
Hyperlipidemia	0.147	

BMI, body mass index; UA, uric acid; eGFR, estimated glomerular filtration rate.

independent risk factors for HUA in the DKD population. The characteristic variables, such as gender, family history of T2DM, drinking history, BMI, eGFR, and hyperlipidemia, were screened as predictors for the HUA risk model by LASSO combined with 10-fold cross-validation. We then validated the risk prediction model in terms of discrimination, fitting degree, and clinical applicability. In the training and validation cohorts, the C-index was 0.761 (95% CI: 0.712–0.810) and 0.843 (95% CI: 0.780–0.906), respectively; the DCA showed that the participants could benefit when the risk probability thresholds were 9–79% and 7–100%; meanwhile, the risk model passed the Hosmer–Lemeshow test with a high goodness of fit.

The proportion of HUA among DKD patients was found to be 32.4% in the study, higher than the 13% in Zhengzhou, China (27), which might be related to the region as well as the inclusion of the study population. Current research in this area is still limited and more studies are necessary in the future. The relationship between DKD and HUA is complex, causally indistinguishable, and mutually reinforcing (28), and the prevailing view is that UA is a modifiable and independent risk factor for chronic kidney disease (29). In contrast, we identified independent risk factors associated with HUA based on the DKD population. Obesity as a risk factor for HUA has been proven in several studies (30–32). The accumulation of visceral fat in obese people affects the metabolic capacity of the kidneys, thus inhibiting the excretion of UA (33, 34). Our research showed that hyperlipidemia is a risk factor for HUA, which was supported by a previous study (35). Although the cause of the increased prevalence of HUA due to lipid metabolism is unknown, potential mechanisms may be related to the metabolic pathways of free fatty acids (36). A chronic hyperglycemic state stimulates the pancreas to produce insulin overload, and elevated insulin promotes UA reabsorption by the proximal renal tubules (37). Therefore, a higher HbA1c often means a higher

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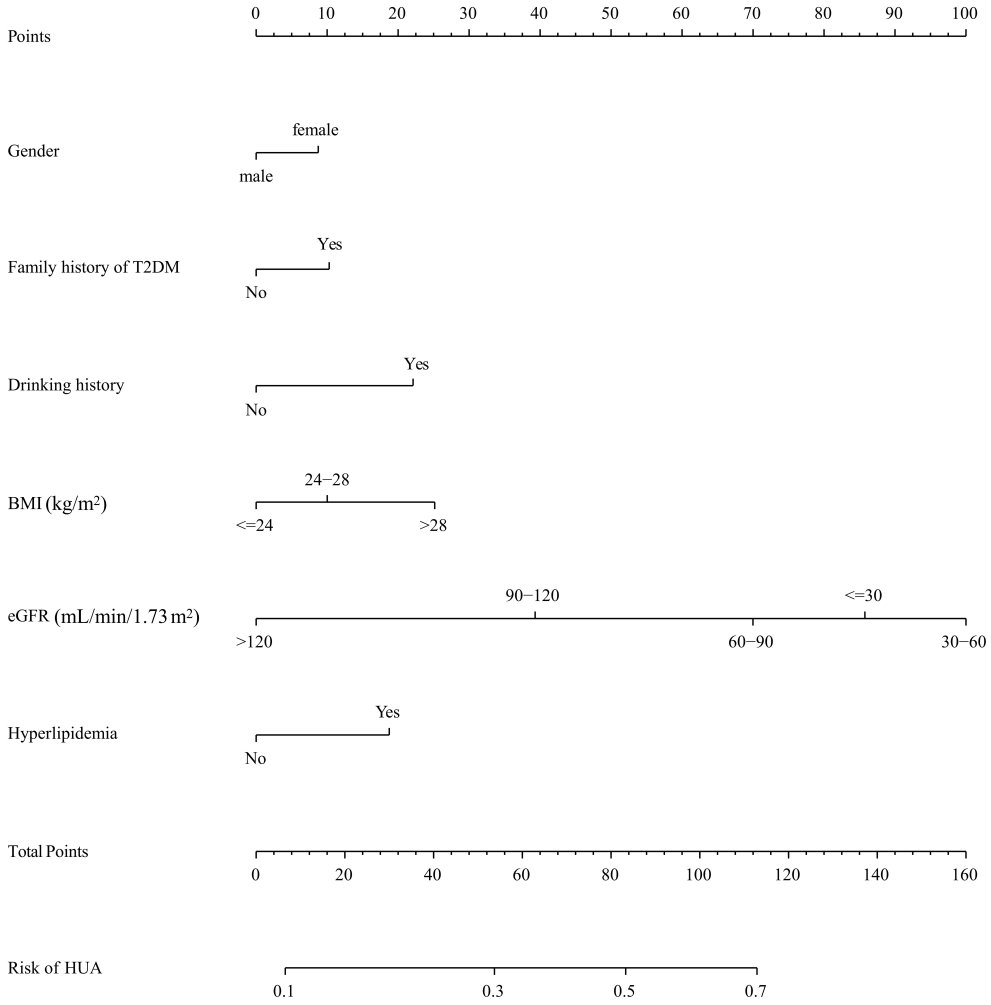

FIGURE 3
A nomogram for predicting the probability of developing HUA in DKD population. The nomogram is used by scoring each variable on its corresponding score scale. The scores for all variables are then summed to obtain the total score, and a vertical line is drawn from the total point row to indicate the estimated probability of the development of HUA in DKD population. DKD, diabetic kidney disease; BMI, body mass index; eGFR, estimated glomerular filtration rate.

incidence of HUA (38). In addition, DKD patients already have impaired renal excretion performance, which, together with the above risk factors, would further increase the elevation of UA.

The construction of predictive models is important for the early diagnosis and prevention of diseases. Various HUA risk prediction models have been established in recent years based on normal populations in different regions (11–13, 39), all showing good clinical differentiation. However, the available HUA risk models still have some limitations. Although Cox regression models, artificial neural networks, and random forest models demonstrate good clinical predictive value, the clinical applicability is limited due to their low visualization. Nomograms are often used to visualize risk prediction models due to their simplicity, visualization, and operability. It mainly assigns a value to each predictor based on the regression coefficient and uses the corresponding algorithm to derive a predictive value for the corresponding individual outcome event (40). In addition, previous studies have revealed that the nomogram model outperforms other machine learning models (artificial neural networks and classification tree models) in accuracy and clinical utility (41, 42). In this study, LASSO combined with 10-fold cross-validation screened for characteristic variables associated with HUA, such as gender, family history of T2DM, drinking history, BMI, eGFR, and hyperlipidemia, which are the most readily available variables in clinical practice. The establishment of visual predictive models can better contribute to the early diagnosis and prevention of HUA in the DKD population, which is of great significance for countries or regions with relatively scarce medical resources.

Compared to previous studies, we have the following advantages. First, we identified risk factors associated with

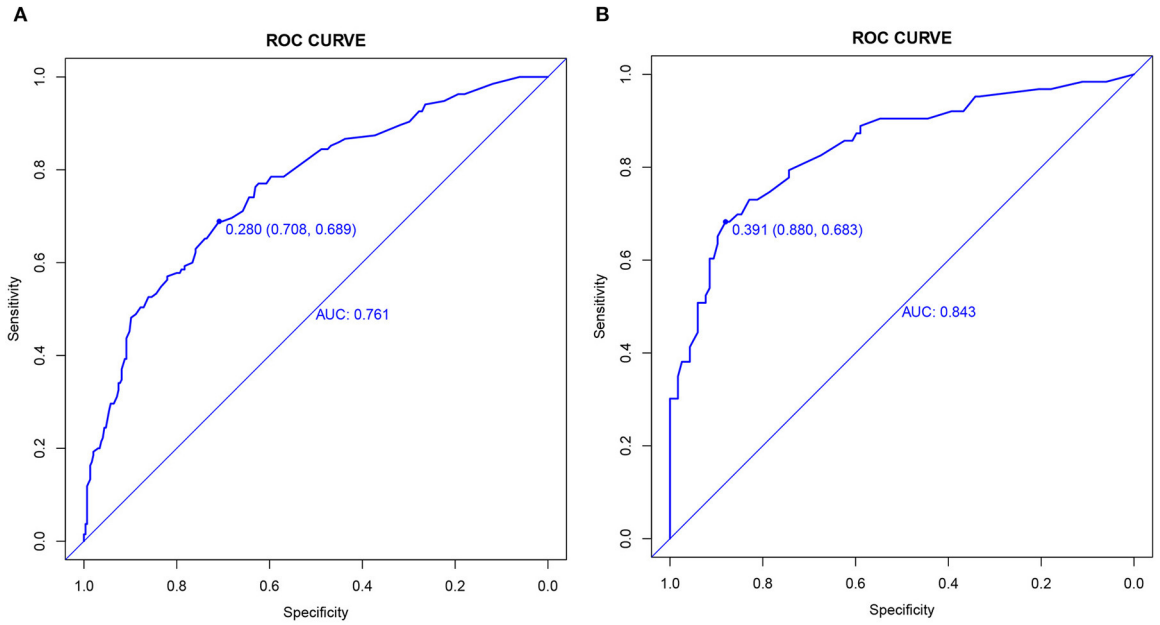

FIGURE 4
ROC curves. **(A)** Training cohort and **(B)** validation cohort.

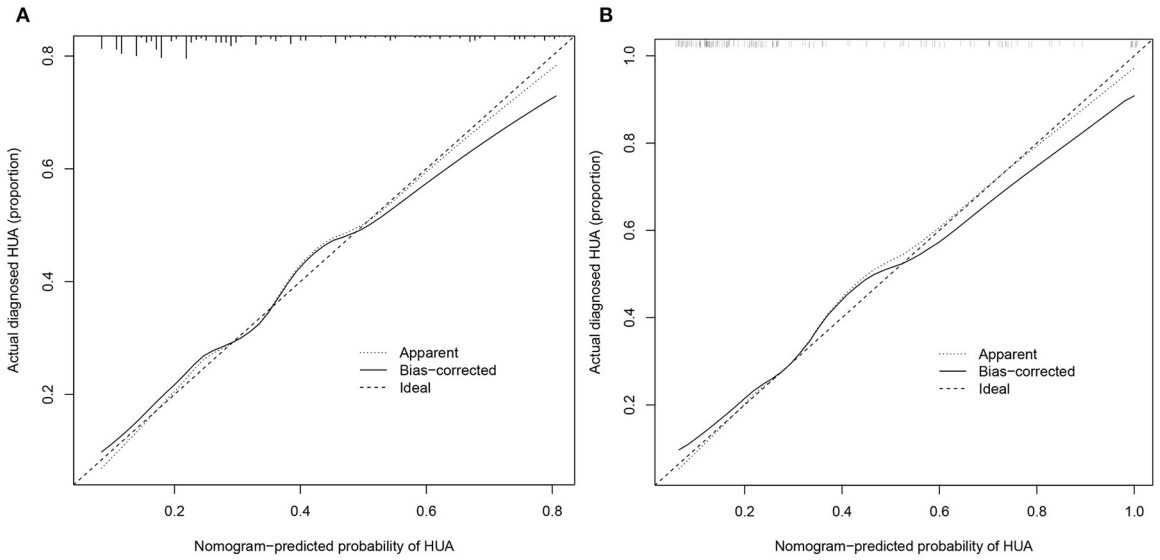

FIGURE 5
Calibration curves. **(A)** Training cohort and **(B)** validation cohort. The x-axis represents the predicted HUA risk. The y-axis represents the actual diagnosed HUA. The diagonal dotted line represents a perfect prediction by an ideal model. The solid line represents the performance of the nomogram, of which a closer fit to the diagonal dotted line represents a better prediction.

TABLE 4 Hosmer–Lemeshow test.

	χ^2	*P*-Value
Training cohort	3.696	0.930
Validation cohort	7.456	0.590

HUA based on the DKD population, which has rarely been reported. HUA is well known as a risk factor for DKD, while the identification of HUA risk factors could help delay the progression of DKD and reduce the occurrence of adverse prognostic events. Second, we may be the first to develop an HUA risk prediction model based on a DKD population,

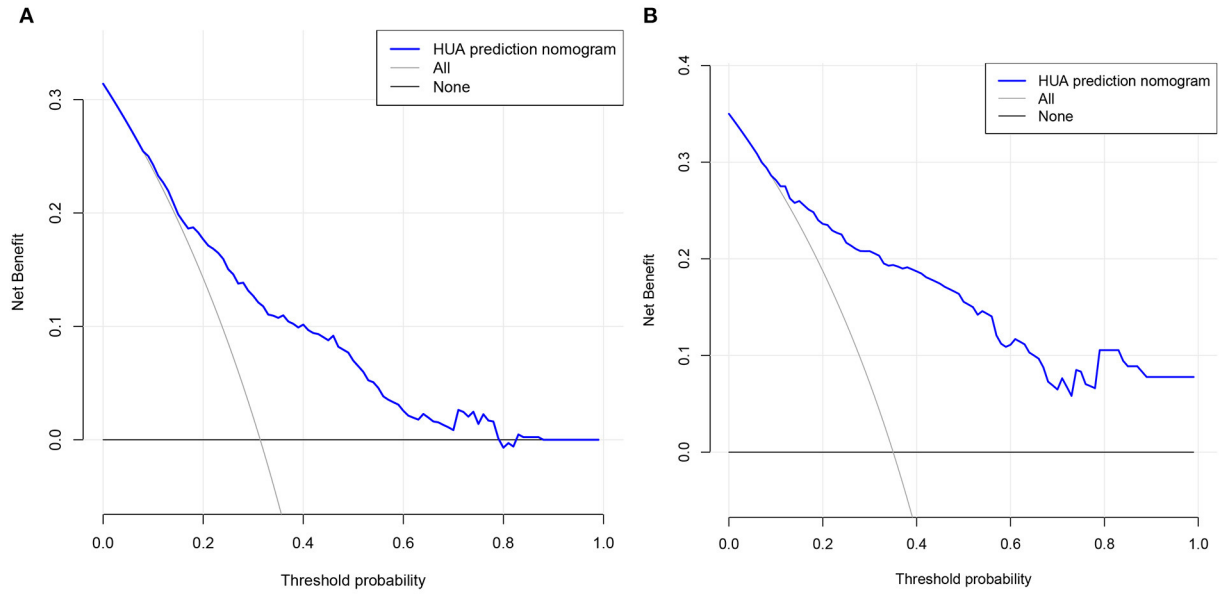

FIGURE 6
Decision curve analysis. **(A)** Training cohort and **(B)** validation cohort. The black line represents the net benefit when none of the participants are considered to develop HUA, while the light gray line represents the net benefit when all participants are considered to develop HUA. The area between the blue line and light gray line in the model curve indicates the clinical utility of the model.

which has important implications for the early diagnosis and prevention of the disease. Certainly, there are some limitations in our study. First, the diagnosis of DKD is predominantly clinical, so the presence of nondiabetic kidney disease cannot be completely ruled out. Second, as a cross-sectional study, there is no escape from the fact that our sample size was limited. Third, the HUA risk prediction model is only validated by internal datasets, while the validation of external datasets is necessary. Furthermore, we will expand the sample size to improve the stability of the model; meanwhile, we will cooperate with multiple centers to obtain external datasets to validate the model.

Conclusions

Briefly, based on a multicenter study in Ningbo, China, we identified independent risk factors (BMI, SBP, eGFR, and hyperlipidemia) associated with HUA and constructed an HUA risk prediction model in the DKD population. The establishment of risk prediction helps us to identify individuals at high risk of HUA early in the DKD population, which is important for the prevention and reduction of adverse prognostic events in DKD.

Data availability statement

The original contributions presented in this study are included in the article, further inquiries can be directed to the corresponding author/s.

Ethics statement

The study was reviewed and approved by the Ethics Committee of the Affiliated Hospital of Medical School, Ningbo University, Ningbo, China. The patients/participants provided their written informed consent to participate in this study.

Author contributions

GH and ML conceived and designed the research, drafted the manuscript, and took part in the discussion. GH performed the statistical analysis. YM and YL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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A systematic review and meta-analysis of gut microbiota in diabetic kidney disease: Comparisons with diabetes mellitus, non-diabetic kidney disease, and healthy individuals

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Background: Gut microbiota has been reported to play an important role in diabetic kidney disease (DKD), however, the alterations of gut bacteria have not been determined.

Methods: Studies comparing the differences of gut microbiome between patients with DKD and non-DKD individuals using high-throughput sequencing technology, were systematically searched and reviewed. Outcomes were set as gut bacterial diversity, microbial composition, and correlation with clinical parameters of DKD. Qualitative data were summarized and compared through a funnel R script, and quantitative data were estimated by meta-analysis.

Results: A total of 15 studies and 1640 participants were included, the comparisons were conducted between DKD, diabetes mellitus (DM), non-diabetic kidney disease (NDKD), and healthy controls. There were no significant differences of α-diversity between DKD and DM, and between DKD and NDKD, however, significant lower microbial richness was found in DKD compared to healthy controls. Different bacterial compositions were found between DKD and non-DKD subjects. The phylum *Actinobacteria* were found to be enriched in DKD compared to healthy controls. At the genus level, we found the enrichment of *Hungatella*, *Bilophila*, and *Escherichia* in DKD compared to DM, patients with DKD showed lower abundances of *Faecalibacterium* compared to those with NDKD. The genera *Butyricicoccus*, *Faecalibacterium*, and *Lachnospira* were depleted in DKD compared to healthy controls, whereas *Hungatella*, *Escherichia*, and *lactobacillus* were significantly enriched. The genus *Ruminococcus torques group* was

demonstrated to be inversely correlated with estimated glomerular filtration rate of DKD.

Conclusions: Gut bacterial alterations was demonstrated in DKD, characterized by the enrichment of the genera *Hungatella* and *Escherichia*, and the depletion of butyrate-producing bacteria, which might be associated with the occurrence and development of DKD. Further studies are still needed to validate these findings, due to substantial heterogeneity.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42022340870.

KEYWORDS

gut microbiota, diabetic kidney disease, diabetes mellitus, systematic review, meta-analysis

Introduction

Diabetic kidney disease (DKD) or diabetic nephropathy (DN), is one of the most common microvascular complication of diabetes mellitus (DM), characterized by progressive renal impairment and albuminuria (1). The condition is a major cause of chronic kidney disease (CKD) and end-stage kidney disease (ESKD), and is associated with higher risk of cardiovascular events and all-cause mortality in diabetic patients (2). Data from the United States Renal Data System indicated that DKD was the leading attributable cause of ESKD, accounting for 46.6% in 2019 (3). Numerous efficacious therapies have been successfully administrated for DKD and have shown renal benefits, such as renin-angiotensin system (RAS) inhibitors, sodium-glucose cotransporter-2 inhibitors, incretin-based therapeutic agents, and finerenone (4); however, substantial residual risk of irreparable renal failure remains (5). Given that the pathological mechanism of DKD has not yet been elucidated, more understanding of the pathogenesis of DKD is urgent for its prevention and treatment. Gut microbiome is relatively stable and participates in various physiological processes (6). However, gut dysbiosis, characterized by imbalance of gut bacterial composition, was found to be associated with the onset and progression of numerous chronic diseases (7). Recently, mounting evidence supports the important role of gut microbiota and their metabolites in diabetes and DKD (8). Excess acetate produced by gut dysbiosis has been shown to be involved in renal injury by activating intrarenal RAS (9), and contributed to tubulointerstitial injury through regulating cholesterol homeostasis *in vivo* and *in vitro* (10). Gut microbiota depletion mediated by antibiotic and faecal microbiota transplantation attenuated glomerular injury and stabilized metabolic homeostasis (11). Dietary fiber showed renoprotective effects of relieving albuminuria and attenuating glomerular injury and interstitial fibrosis, through reshaping gut microbial ecology and promoting the expansion of short-chain fatty acid (SCFA)-producing bacteria in diabetic mice (12). Patients with DN receiving supplementation of probiotics for 12 weeks showed significantly lower serum creatinine and albuminuria than those receiving placebo (13, 14). Given the potential pathogenic role of intestinal dysbiosis in DKD according to recent evidence, characterizing the gut microbiota in DKD might be beneficial for formulating therapeutic strategy. Previous investigations have reported the existence of gut dysbiosis in patients with DKD compared to healthy volunteers, including the changes bacterial diversity and alterations of microbial composition, however, their findings were inconsistent (15). Additionally, the differences of gut microbiota between DKD and DM or non-diabetic kidney disease (NDKD) were also not determined. This systematic review was designed to compare the differences of microbial diversity and bacterial composition between patients with DKD and non-DKD individuals, aiming to characterize the alterations of gut bacteria in DKD and provide potential microbiota targets for the intervention of DKD.

Materials and methods

Registration and statement

This systematic review was pre-registered in International Prospective Register of Systematic Reviews (PROSPERO, CRD42022340870) and performed in accordance with the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Checklist, Supplementary Table 1) (16).

Search strategy

The literature search was conducted on PubMed, Embase, Web of Science, China national knowledge infrastructure, Cochrane Library, and *ClinicalTrials.gov*, from inception until 3 July 2022. Eligible studies comparing gut microbiota between patients with DKD and non-DKD persons were retrieved using the search terms with DKD, gut microbiota, and their relevant keywords. Our detailed searching strategies for each database is detailed in Supplementary Table 2.

Eligible criteria and outcomes

Studies comparing the diversity and/or composition of gut microbiota between patients with DKD and non-DKD individuals using high-throughput sequencing technology, were included. The inclusion criteria and exclusion criteria according to the PICOS principle are shown in Table 1. The primary outcome was gut microbial diversity, and the secondary outcomes were gut microbial composition and the correlations between clinical parameters of DKD and specific bacteria. In this review, we compared α-diversity and β-diversity between different groups. Bacterial α-diversity was evaluated by observed species/Chao1/ACE-based richness index and Shannon/Simpson-based community diversity index. β-diversity represents the differences of gut microbial structure between DKD and non-DKD individuals.

Study selection, data extraction, and quality assessment

After removal of duplicates, two reviewers screened titles and abstracts of the retrieved records independently (S.H. and P.C.), and disagreements were solved by discussing with a third researcher (Y.X.). The full texts were screened for eligible studies by Y.L. and Y.W. For each included study, two reviewers (Z.Z. and M.C.) extracted the following data independently: author, publication year, country, study design, diagnostic criteria of DKD, characteristics of all groups, including sample size, age, sex, matched factors, serum creatinine, estimated glomerular filtration rate (eGFR), urinary albumin-creatinine ratio (UACR), and urinary total protein (UTP), stool sample collection and storage, DNA extraction method, sequencing platform, bioinformatics pipelines, and outcomes. Methodological quality was assessed using the Newcastle-Ottawa Scale (NOS) for case-control study and the modified version for cross-sectional study. The NOS scale has three domains for evaluation: selection, comparability, and exposure/outcome, maximizing 9 scores for case-control study and 7 scores for cross-sectional study. A total score of ≥ 7 for case-control studies and ≥ 4 scores for cross-sectional studies were considered as high quality (17).

Statistical analysis

The quantitative and qualitative data of gut microbiota diversity and relative abundance between DKD and non-DKD individuals reported in each study were record, and were synthesized by qualitative summary and meta-analysis, respectively. For qualitative analysis, the results of each prespecified outcome were summarized and presented as stacked histograms. A funnel R script was adopted to explore differential bacteria between different groups at the significance levels of 80% and 95%, through calculating a binomial Poisson distribution score2 (18). For meta-analysis, standardized mean difference (SMD) and 95% confidence intervals (CIs) were calculated to evaluate the differences in diversity indices and relative abundances of gut bacteria between DKD and non-DKD groups. Heterogeneity was quantified using Cochrane I^2 test, which was considered significant when $I^2 > 50\%$ (19). Meta-analysis was then conducted to estimate pooled SMD using a fixed-effects model or a random-effects model according to heterogeneity. Sensitivity analysis and subgroup analysis were performed according to different inclusion criteria of DKD. All the statistical processes and results visualization were conducted

TABLE 1 Eligible criteria based on PICOS.

	Inclusion criteria	Exclusion criteria
Participant	Patients with DKD diagnosed clinically or biopsy-proven DN	Patients receiving dialysis; patients with diabetes and other chronic kidney disease
Exposure	DKD/DN	–
Comparator	DKD vs. Non-DKD, including diabetes mellitus, non-diabetic kidney disease, and healthy controls	–
Outcomes	Gut microbial diversity and composition	Insufficient data for analysis; not high-throughput sequencing technology for detecting gut microbiome
Study design	Observational study	–

DKD, diabetic kidney disease; DN, diabetic nephropathy.

by Stata (version 14.0), RStudio (Open source, version 2021.9.2 + 382), and GraphPad Prism (version 8.0).

Results

Study characteristics

According to our retrieval strategy, a total of 8618 records were searched from the electronic databases and registers. After removing duplicates and screening titles and abstracts, 8569 publications were excluded. Ultimately, 15 studies were selected according to the inclusion criteria and exclusion criteria during full-text screen. The study selection process and reasons for exclusion are shown in Supplementary Figure 1.

The characteristics of included studies in this review are presented in Table 2, including 15 cross-sectional studies published from 2019 to 2022 (20–34). One study was conducted in Denmark (22), and the other fourteen studies were completed in China. Two studies included patients with biopsy-proven DN (20, 31), and the remaining 13 studies enrolled patients with DKD who were diagnosed clinically. Eight studies compared the differences of gut microbiota among patients with DKD, patients with DM, and healthy volunteers (20, 21, 24, 25, 28, 30, 32, 34), four studies reported the differences of intestinal microbiota between DKD and healthy controls (22, 26, 29, 31), one study conducted the comparison between DKD and type 2 DM (33), and the other two studies analyzed the differences of gut bacteria between patients with DKD and those with NDKD (23, 27). All the included studies stated that they have excluded subjects with gastro-intestinal or systemic diseases known to affect gut microbiota, and those taking antibiotics or prebiotics/probiotics within 1 to 3 months before enrollment. According to the included studies, 830 fecal specimens were collected from patients with DKD, 514 from healthy volunteers, 256 from diabetic individuals, and 40 from patients with NDKD. All the enrolled studies reported that fresh stool samples were collected and stored at −80°C until DNA extraction, and 16S ribosomal gene amplicon sequencing was adopted for gut microbiota analysis. The amplified region was V3-V4 in eight studies (20, 23, 25–27, 29, 32, 34), V3 in one study (24), and V4 in four studies (21, 22, 28, 30), two studies did not report the amplified region (31, 33). Illumina sequencing platform was adopted in 14 studies, while only one study used the Ion S5TM platform (32).

Six studies were awarded seven scores according to the modified NOS scale for cross-sectional studies, because of adequate selection for subjects, sufficient ascertainment of outcome, and controls of at least two confounding factors (20–22, 31, 33, 34). Three studies were assessed for six scores, because there were only one factor were matched between cases and controls (23, 29, 32). Six studies were given six scores, due to the absence of detailed diagnostic criteria of DKD (24–28, 30).

Bacterial diversity

The purpose of this review was to explore the alterations of gut microbiota in patients with DKD. According to the existing evidence, the comparisons of intestinal bacteria were carried out between DKD and DM, DKD and healthy control, and DKD and NDKD, respectively.

The qualitative comparisons of microbial diversity indices between patients with DKD, diabetes individuals, and healthy controls are presented in Figure 1A. Three of four studies reported non-significantly changes of observed species (21, 25, 28) and ACE index (20, 30, 34) between the DKD and DM groups, while Tao et al. (20) and Cai et al. (32) reported an increased indices of observed species and ACE in patients with DKD, respectively. The Chao1 index was found to be significantly higher in patients with DKD than diabetes patients in one study (32), lower in one study (28), and not significantly changed in four studies (20, 25, 30, 34). Six (20, 25, 28, 30, 32, 34) and five (20, 28, 30, 32, 34) studies reported that there were no significant differences between DKD and DM in Shannon and Simpson index. Two studies reported the differences of gut microbiome in α-diversity between DKD and NDKD patients (23, 27). Opposite results were shown in the observed species and Shannon index. For Chao1, ACE and Simpson index, one study suggested that they were significantly higher in patients with DKD than in those with NDKD, while another study showed non-significant differences.

Compared with healthy controls, significant lower observed species (21, 26–28) and ACE index (20, 26–28) of gut microbiome in patients with DKD were found in four studies, whereas other three studies reported unchanged proportion (20, 22, 29, 30, 32, 34). Two (26, 28) and six studies (20, 25, 27, 30, 32, 34) reported significantly lower and non-significant alterations of the Chao1 index in DKD patients compared to healthy volunteers, respectively. Shannon index was shown to be significantly higher in DKD patients in one study (31), lower in one study (27), and not changed in eight studies compared to healthy participants (20, 22, 25, 26, 28, 30, 32, 34). For the Simpson index, the number of studies reporting a significant increase (27, 29), a significant decrease (32), and non-significant change in DKD groups compared to healthy groups (20, 22, 26, 28, 30, 34), were 2, 1, and 6, respectively.

Based on the available data of α-diversity index, we conducted a quantitative meta-analysis (Figure 1B). The results showed that there were no statistical differences in α-diversity indices of gut bacteria between DKD and DM patients, as well as those between DKD and NDKD individuals. Compared to healthy volunteers, patients with DKD showed significantly lower microbial richness

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TABLE 2 Characteristics of the included study.

Study	Location	Comparisons	Eligible criteria of DKD/DN	Case [n (female %, age)]	Control [n (female %, age)]	Matched factors	Sequencing platform (Region)	Database	Outcomes	Modified NOS score
Tao 2019 (20)	Guangdong, China	DN vs. T2DM vs. HC	Biopsy-proven DN, eGFR ≥ 60 mL/min/1.73 m^2 and UACR ≥ 30 mg/g	14 (36%), 52.93 ± 9.98	T2DM: 14 (36%), 53.29 ± 9.00; HC: 14 (36%), 52.86 ± 9.91	Age, sex, BMI	Illumina MiSeq (V3-V4)	RDP, Silva	α-diversity; β-diversity; microbial composition; clinical correlation	7
Bao 2019 (21)	Sichuan, China	DKD vs. T2DM vs. HC	DM complicated with massive proteinuria; or with DR and CKD; microalbuminuria in T1DM of more than 10 years	25 (36%), 63.7 ± 13.3	T2DM: 30 (54%), 62 ± 13.3; HC: 30 (47%), 60.2 ± 9.7	Age, sex	Illumina TruSeqTM (V4)	Greengenes	α-diversity; β-diversity; microbial composition	7
Winther 2020 (22)	Copenhagen, Denmark	T1DKD vs. HC	T1DM with eGFR ≥ 15 mL/min/1.73 m^2 and excluded non-diabetic kidney disease	161 (42%), 60 ± 10	50 (44%), 59 ± 13	Age, sex, BMI	Illumina HiSeq2500 (V4)	Dada2 R package	α-diversity; β-diversity; microbial composition; clinical correlation	7
Yu 2020 (23)	Henan, China	DKD vs. MN	Diabetes more than 5 years with UACR ≥ 30 mg/g and presence of DR	129 (36%), 56 (49, 65)	142 (36%), 49 (43, 56)	Sex	Illumina MiSeq (V3-V4)	RDP, Silva	α-diversity; β-diversity; microbial composition	6
Feng 2020 (24)	Sichuan, China	DKD vs. T2DM vs. HC	Clinically diagnosed DKD and not on dialysis	57 (41%), 55.23 ± 11.21	T2DM: 68 (47%), 54.36 ± 11.12; HC: 36 (42%), 54.84 ± 11.17	Age, sex	Illumina HiSeq 2500 (V3)	NR	β-diversity; microbial composition	6
Chen 2021 (25)	Beijing, China	DKD vs. DM vs. HC	DKD with UAER ≥ 30 mg/24h or UACR ≥ 30 mg/g	60 (32%), 60.53 ± 9.62	DM: 20 (40%), 55.2 ± 14.77; HC: 20 (60%), 55.15 ± 13.77	Age, BMI, diet	Illumina MiSeq (V3-V4)	RDP, Silva	α-diversity; microbial composition; clinical correlation	6
Du 2021 (26)	Tianjin, China	DKD vs. HC	DN stage 3 or 4, without detailed criteria, not uremia	43 (26%), 60.86 ± 5.69	37 (33%), 61.78 ± 6.40	Age, sex, BMI	Illumina MiSeq (V3-V4)	RDP, Silva	α-diversity; β-diversity; microbial composition	6
Sun 2021 (27)	Shandong, China	DKD vs. NDKD vs. HC	Clinically diagnosed DKD	25 (36%), 62.52 ± 13.61	NDKD: 40 (23%), 53.98 ± 13.81; HC:24 (34%), 56 ± 9	Age, sex	Illumina MiSeq (V3-V4)	RDP, Silva	α-diversity; β-diversity; microbial composition	6
Song 2021 (28)	Inner Mongolia, China	DKD vs. T2DM vs. HC	Diagnosed DKD	20 (40%), 58.2 ± 9.4	T2DM: 20 (50%), 54.1 ± 13.5 HC: 20	Age, sex	Ilumina, NovaSeq PE250 (V4)	Dada2 R package	α-diversity; β-diversity; microbial composition;	6

(Continued)

TABLE 2 Continued

Study	Location	Comparisons	Eligible criteria of DKD/DN	Case [n (female %, age)]	Control [n (female %, age)]	Matched factors	Sequencing platform (Region)	Database	Outcomes	Modified NOS score
					(55%), 50.2 ± 12.6				clinical correlation	
Zhang 2021 (29)	Henan, China	DKD/DN vs. HC	Proteinuria or renal impairment caused by diabetes, and other kidney diseases were excluded, meeting one of the following conditions: UACR ≥ 30mg/g or UAER ≥ 30mg/24h or eGFR ≤ 60mL/min/1.73 m^2 or biopsy-proven DN	180 (38%), 55.27 (26-87)	179 (42%), 52.05 (39-69)	SCr	Illumina MiSeq (V3-V4)	RDP	α-diversity; β-diversity; microbial composition; clinical correlation	6
Chu 2021 (30)	Zhejiang, China	T2DKD vs. T2DM vs. HC	DKD with UACR ≥ 30 - 300mg/g or UAER 20-200 ug/min	47 (45%), 69.06 ± 11.23	T2DM: 53 (42%), 68.47 ± 10.82 HC: 42 (43%), 67.11 ± 9.26	Age, sex	Ilumina HiSeq (V4)	NCBI-BLAST	α-diversity; microbial composition	6
Xin 2021 (31)	Shanxi, China	DN vs. HC	Biopsy-proven DN	20 (50%), 55.1 ± 13.83	20 (50%), 50.9 ± 9.49	Age, sex	Ilumina Novaseq6000 (NR)	HUMAnN3	α-diversity; β-diversity; microbial composition; clinical correlation	7
Cai 2022 (32)	Zhejiang, China	DKD vs. T2DM vs. HC	DKD diagnosed clinically: UAER >300 mg/24h and presence of DR, and excluded other kidney diseases	31 (26%), 61.35 ± 10.04	T2DM: 32 (32%), 56.34 ± 10.79; HC: 34 (65%), 56.12 ± 8.11	Age	Ion S5TM (V3-V4)	Ion 530TM Chip	α-diversity; β-diversity; microbial composition	6
He 2022 (33)	Shanxi, China	DKD vs. T2DM	Diagnosed DKD stage 3 or4, presenting normal renal function and UACR > 30 mg/g, renal impairment due to other causes was excluded.	10 (10%), 56.00 ± 14.97	10 (20%), 64.90 ± 7.37	Age, sex, BMI	Illumina HiSeq4000 (NR)	Non-Redundant	β-diversity; microbial composition; clinical correlation	7
Yang 2022 (34)	Guizhou, China	DKD vs. T2DM vs. HC	T2DM with ACR > 265 mg/g or UAER > 300 mg/24h; and/or DR with ACR 22 (male, 31 female) - 265 mg/g; or UAER 30 - 300 mg/24h and/or eGFR < 60 min/mL.	8 (50%), 58.75 ± 7.40	T2DM: 9 (56%), 57.67 ± 4.61; HC: 8 (50%), 57.13 ± 2.8	Age, sex, BMI	Illumina MiSeq (V3-V4)	Greengenes	α-diversity; β-diversity; microbial composition	7

DKD, diabetic kidney disease; DN, diabetic nephropathy; T2DM, type 2 diabetes mellitus; HC, healthy controls; eGFR, estimated glomerular filtration rate; UACR, urinary albumin-creatinine ratio; UAER, urinary albumin excretion rate; NOS, Newcastle-Ottawa Scale; DR, diabetes retinopathy; CKD, chronic kidney disease; BMI, body mass index; SCr, serum creatinine; NR, not reported; RDP, ribosomal database project.

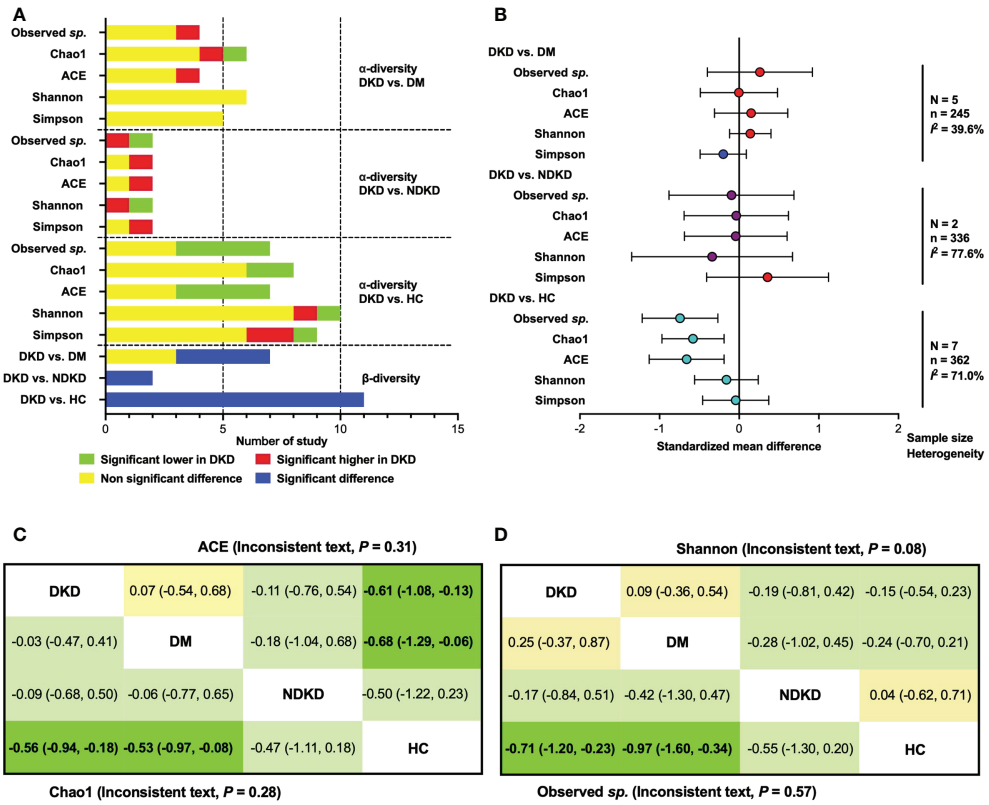

FIGURE 1
Qualitative analysis and meta-analysis for α- and β-diversity. **(A)** Qualitative comparisons for α- and β- diversity; **(B)** Meta-analysis for α-diversity indices. **(C)** Network meta-analysis for ACE and Chao1 index; **(D)** Network meta-analysis for Observed sp. and Shannon index. Data are shown as standardized mean difference (95% confidence interval). The estimate is for the column-defining treatment compared to the row-defining treatment. Statistical significance is defined as 95% CIs that do not overlap zero (bold text). DKD, diabetic kidney disease; DM, diabetes mellitus; HC, healthy controls; NDKD, non-diabetic kidney disease; N, number of study.

index (Observed sp., SMD = -0.74, 95%CI -1.22, -0.27, I^2 = 68.5%; ACE, SMD = -0.66, 95%CI -1.13, -0.19, I^2 = 67.1%; Chao1, SMD = -0.58, 95%CI -0.97, -0.19, I^2 = 67.1%), whereas no significant differences were found in Shannon and Simpson index. Considering that the comparisons were conducted among multiple groups, and the tests for subgroup differences were significant in microbial richness indexes (Supplementary Figure 2), we further performed a random-effects network meta-analysis for α-diversity utilizing previously reported routines (35). No inconsistency was found in the α-diversity indexes, except Simpson index, which showed significant inconsistency (P = 0.02). The results of network comparisons agreed with the above findings, involving observed species, Chao1, ACE, and Shannon index. Additionally, we found that patients with DM also showed lower microbial richness than healthy subjects (Figures 1C, D).

Four studies reported significant differences of β-diversity between DKD and DM (20, 32–34), while three studies showed no significant changes (21, 24, 28). Whether compared with patients with NDKD (23, 27) or healthy controls (20–22, 24, 26–29, 31, 32, 34), significant differences in β-diversity were observed in patients with DKD, indicating fecal microbial alterations in DKD.

Microbial composition at phylum level

Six phyla were reported dominating the gut microbiota, including *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* (Figure 2A). Compared to DM group, *Firmicutes* and *Actinobacteria* were found to be depleted in DKD group in one study (20), whereas eight studies reported non-significant differences (21, 24, 25, 28, 30, 32–34). The relative abundances of *Proteobacteria* were shown to be enriched in patients with DKD compared to diabetic persons in three studies (20, 32, 33), while six studies did not find any difference between the two groups (21, 24, 25, 28, 30, 34). The proportions of *Bacteroidetes*, *Fusobacteria*, and *Verrucomicrobia* did not show differences between DKD and DM according to the results of nine studies (20, 21, 24, 25, 28, 30, 32–34). Only two studies compared gut microbiota between DKD and NDKD at phylum level, their results

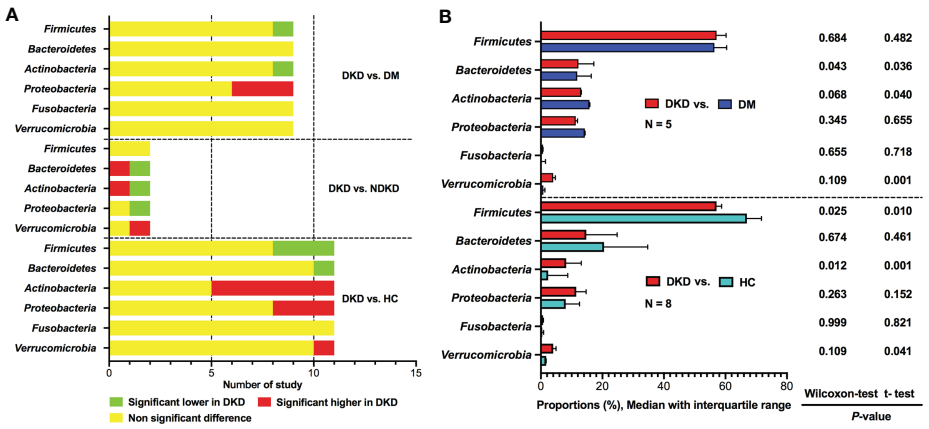

FIGURE 2
Qualitative and quantitative analysis of gut microbiota at the phylum level. **(A)** Qualitative comparisons at the phylum level; **(B)** Comparisons of average abundances at the study level for bacterial phylum. DKD, diabetic kidney disease; NDKD, non-diabetic kidney disease; DM, diabetes mellitus; HC, healthy controls; N, number of study.

showed that the abundances of *Firmicutes* were similar between the two groups, while the comparisons of *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* showed inconsistent results (23, 27).

Eleven studies reported the relative abundances of bacterial phyla between patients with DKD and healthy controls (20, 21, 24–30, 32, 34). Three studies showed decreased abundances of *Firmicutes* in DKD (21, 28, 32), whereas eight studies reported non-significant differences between patients with DKD and healthy volunteers (20, 24–27, 29, 30, 34). For *Bacteroidetes*, only one studies found that it was depleted in DKD group (29), while ten studies showed that the *Bacteroidetes* taxa was not statistically different between DKD patients and healthy individuals (20, 21, 24–28, 30, 32, 34). *Actinobacteria* was found to be higher in patients with DKD than those in healthy controls in six studies (24–27, 29, 34), however, five studies indicated non-significant differences (20, 21, 28, 30, 32). Regarding *Proteobacteria*, three studies supported increased abundances in DKD (27, 29, 32), while the remaining eight studies did not find differences between DKD and healthy controls (20, 21, 24–26, 28, 30, 34). None of the included studies reported differences in *Fusobacteria* between DKD and healthy controls. *Verrucomicrobia* was reported to be enriched in patients with DKD in one study (29), however, no significant changes were observed in ten studies (20, 21, 24–28, 30, 32, 34).

Due to the limited data, we can only calculate the differences in the average abundances of bacterial phyla between DKD and non-DKD individuals at the study level (Figure 2B). Compared with diabetic population, patients with DKD might have mildly increased taxa of *Bacteroidetes*. The average abundance of *Firmicutes* was found to be lower in patients with DKD than that in healthy controls, whereas *Actinobacteria* was significantly enriched in DKD patients, which was consistent with the results of qualitative analysis.

Microbial composition at genus level

Eight studies reported the differences of gut bacteria between DKD and DM at the genus level (20, 21, 24, 25, 28, 32–34). The genera that were reported to be statistically different between the two groups in two or more studies are presented in Figure 3A. *Hungatella* was shown to be enriched in DKD compared to DM in three studies (20, 28, 32), *Bilophila* and *Escherichia* were found to have higher proportions in DKD patients in two studies (20, 33). The proportion of studies reporting significantly higher or lower abundances of specific genera were compared using a funnel R script, which also suggested that the genera *Hungatella*, *Bilophila*, and *Escherichia* might be the differential bacteria between DKD and DM (Figure 3B). When comparing the genera between DKD and NDKD, we found that only *Faecalibacterium* had consistent results in the two studies, that is, it was depleted in DKD patients (Figure 3A) (23, 27). Twelve studies presented the comparisons of gut microbiome between DKD and healthy individuals at the genus level (20–22, 24–29, 31, 32, 34). *Faecalibacterium* (21, 22, 26, 27, 29, 31), *Lachnospira* (21, 22, 25, 27, 31, 32), *Roseburia* (21, 25–27, 31), and *Butyricicoccus* (22, 26, 27, 32) were reported to be depleted in DKD in at least four studies, whereas *Hungatella* (20, 22, 28, 31, 32), *Lactobacillus* (21, 22, 26, 27, 29), and *Escherichia* (20, 27, 29, 31) were found to be enriched in DKD in five or four studies (Figure 3C). The funnel plot indicated that the genera *Hungatella* were enriched in DKD, whereas *Butyricicoccus*, *Faecalibacterium*, and *Lachnospira* were depleted (Figure 3D).

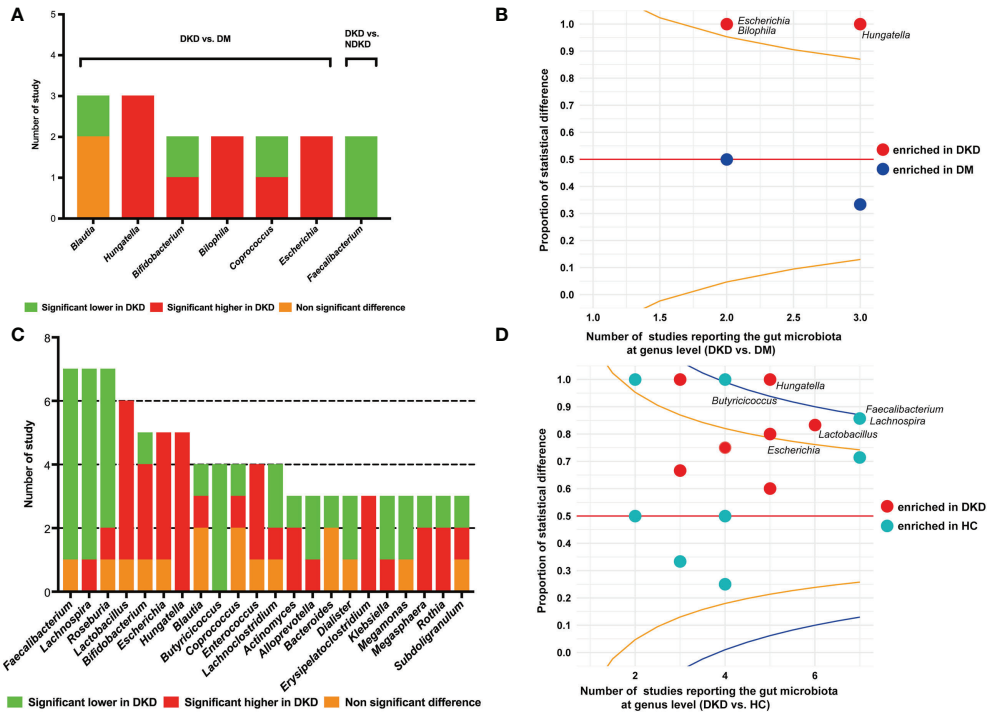

FIGURE 3
Comparisons of gut microbiota at the genus level. **(A)** The genera reported to be statistically different between DKD and DM, and between DKD and NDKD; **(B)** The funnel plot conducted between DKD and DM, specified score2 confidence limits were showed at 80% (orange line) and 95% (blue line); **(C)** The genera reported to be statistically different between DKD and HC; **(D)** The funnel plot conducted between DKD and HC. DKD, diabetic kidney disease; NDKD, non-diabetic kidney disease; DM, diabetes mellitus; HC, healthy controls.

Only two studies detailed the abundances of gut microbiota between DKD patients and healthy volunteers at genus level. Meta-analysis suggested that *Bifidobacterium* (SMD = 5.25, 95%CI 3.47, 7.03, I^2 = 76.3%) and *Lactobacillus* (SMD = 4.05, 95%CI 2.95, 5.14, I^2 = 64.7%) had higher relative proportion in DKD patients compared to healthy persons, while *Roseburia* (SMD = -3.25, 95%CI -4.01, -2.49, I^2 = 44.7%) was enriched in healthy volunteers (Figure 4). The results of *Lactobacillus* and *Roseburia* were consistent with that from qualitative analysis. However, this result should be interpreted with caution, due to the limited data and substantial heterogeneity.

Correlation of gut microbiota and clinical parameters of DKD

The phyla and genera of gut bacteria with statistical correlation with clinical parameters of DKD were recorded, including UACR, UTP, eGFR, and serum creatinine. Three phyla and thirty-four genera were reported to have a positive or negative association with proteinuria or renal function in at least one study (Figure 5). In particular, Three studies supported a negative correlation between the genus *Ruminococcus torques group* (*R. torques*) and eGFR in patients with DKD. Two studies reported that *Hungatella* was positively correlated with serum creatinine and negatively correlated with eGFR, suggesting the harmful effect of *Hungatella* on aggravating kidney injury of DKD (28, 31).

Sensitivity analysis and subgroup analysis

Considering that the definition of DKD was not consistent across the enrolled studies, involving clinically diagnosed DKD and biopsy-proven DN, we conducted sensitivity analysis and subgroup analysis to test the stability of the results and compare the differences of gut bacteria between different inclusion criteria. When biopsy-proven DN was excluded, the results based on patients with clinically diagnosed DKD were consistent with the findings from the qualitative and quantitative analyses of all the included 15 studies (Supplementary Figures 3A–C, E, F).

The subgroup of biopsy-proven DN consisted of two studies and 82 participants (20, 31). Detailed α-diversity index was reported in one study (20), indicating higher observed species in biopsy-proven DN than those in DM, and a lower ACE index in DN group compared to healthy controls (Supplementary

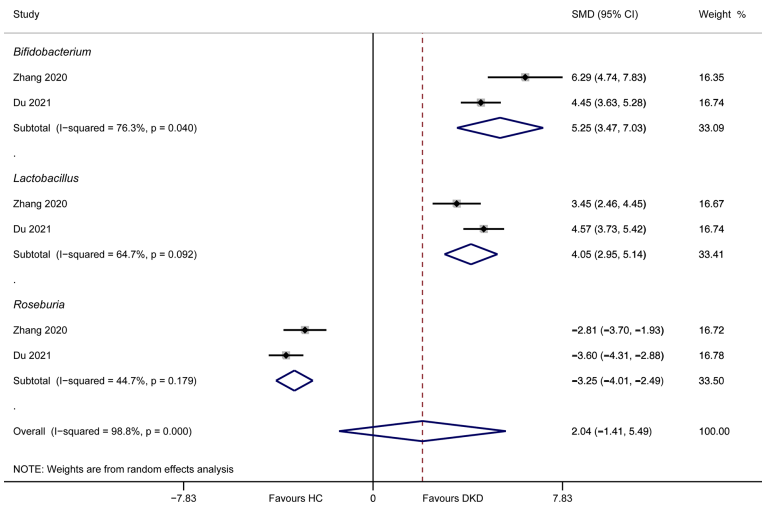

FIGURE 4
Meta-analysis of the genera between DKD and HC. DKD, diabetic kidney disease; HC, healthy controls; SMD, standardized mean difference; CI, confidence interval.

Figure 3D). At the genus level, three genera were found predominantly in biopsy-proven DN versus DM, including *Hungatella*, *Escherichia*, and *Bilophila*. Moreover, *Hungatella* and *Escherichia* were still identified to be enriched in DN group when compared to healthy controls (Supplementary Figures 3G, H). These changes in bacterial composition were consistent with the findings from clinically diagnosed DKD, as well as the results from all of the 15 studies, suggesting the potential important roles of *Hungatella* and *Escherichia* in DKD.

Discussion

Accumulating evidence has demonstrated that alterations of composition and function in gut microbiota were correlated with increased risk of the occurrence and development of diabetes and its associated complications (36). This review was designed to comprehensively characterize the alterations of gut microbiome in DKD, by comparing with diabetes, NDKD, and healthy controls. A total of 15 cross-sectional studies and 1640 participants were

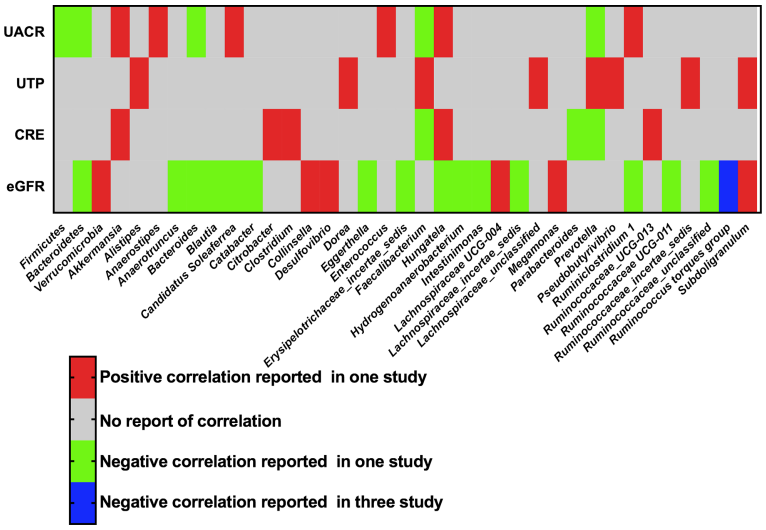

FIGURE 5
Correlation of gut microbiota and clinical parameters of DKD. UACR, urinary albumin-creatinine ratio; UTP, urinary total protein; CRE, creatinine; eGFR, estimated glomerular filtration rate.

included for comparison. There was no significant difference in the α-diversity of gut microbiota between DKD and diabetes subjects, as well as the comparison between DKD and NDKD. Lower microbial richness indices were found in DKD patients compared to healthy volunteers. Unlike α-diversity, β-diversity analysis suggested significant microbial differences between DKD and NDKD and healthy controls; four of seven studies showed significant differences in β-diversity between DKD and DM individuals. At the phylum level, *Actinobacteria* was found to be enriched in DKD compared to healthy controls, however, no significant difference was found when comparing with DM. *Actinobacteria* was closely related to the metabolism of trimethylamine-N- oxide (TMAO), high levels of circulating TMAO were demonstrated to contribute to renal dysfunction through promoting inflammation (37), oxidative stress, and fibrosis (38). Patients with DKD had a significantly higher level of TMAO than those with diabetes, moreover, TMAO also showed positive correlation with UACR (34). At the genus level, *Hungatella*, *Bilophila*, and *Escherichia* showed higher abundances in DKD compared to DM, and *Faecalibacterium* was found to be depleted in DKD compared to NDKD. The genera *Hungatella*, *Bilophila*, and *Escherichia* are all gram-negative, recognized by their pathogenic and infectious potential, since many members are opportunistic pathogens that induce inflammation and disrupt gut barrier function (39–41). Interestingly, patients with type 2 diabetes receiving empagliflozin showed similar gut microbiota alterations with our findings, accompanying with improved glucose metabolism and decreased interleukin-6 (IL-6), that is, depleted taxa of the harmful bacteria of *Escherichia*, *Bilophila*, *and Hungatella*, and enrichment of SCFA-producing bacteria, such as *Roseburia* and *Faecalibacterium* (42). *Hungatella* was reported as a TMAO-producer (41), whereas *Bilophila* is a sulfate-reducing bacteria, which have pro-inflammatory effects and have been shown to be associated with a variety of inflammatory or immune diseases, such as diabetes and metabolic syndrome (43). The genus *Escherichia* was found to be enriched in the stool samples of patients with DKD compared to diabetic persons and healthy volunteers in this review. This findings have also been validated in cohorts of CKD, *Escherichia* was identified as the biomarker for the advanced CKD, and the abundance was positively correlated with CKD stages (44). It is documented that *Escherichia* can metabolize tryptophan into indole, which can be converted into indoxyl sulphate (IS) and Kynurenine, and then participate the process of renal impairment (45). IS and Kynurenine have been proved to have renal injury effects, such as promoting endothelial dysfunction (46), inducing tubulointerstitial injury (47), and aggravating renal oxidative stress and inflammation (48). Serum levels of IS and Kynurenine were shown to be positively associated with the progression of DKD (49, 50). *Escherichia* are also conditional pathogens that can enhance gut infiltration through penetrating the intestinal epithelial barrier and aggravate gut leakiness, resulting in the escape of pathogenic and commensal bacteria and subsequent immune responses (51). The enrichment of *Hungatella*, *Escherichia*, and *Lactobacillus* were found in patients with DKD compared to healthy controls, whereas decreased proportions of the genera *Butyricicoccus*, *Lachnospira*, *Faecalibacterium*, and *Roseburia* were indicated according to the qualitative and quantitative analyses. These four genera are butyrate-producing bacteria (52–54), and have been reported diversified renoprotective effects for DKD *in vivo* and *in vitro*, such as improving intestinal barrier function (55), attenuating fibrosis and collagen deposition, inhibiting inflammation (56), and ameliorating TGF-β1-induced fibrogenesis, apoptosis and DNA damage in the diabetic kidney (57). *Lactobacillus* have been used widely in foods and probiotic products and showed beneficial effects (58), however, upregulated inflammatory cytokines were also significantly increased in *Lactobacillus*-treated mice, such as tumor necrosis factor-α, IL-6, and IL-1β (59), therefore, the specific role of *Lactobacillus* in DKD needs to be further studied. Taken together, the alterations of gut microbiome in DKD are mainly manifested as the depletion of beneficial bacteria and enrichment of harmful bacteria and potential pathogenic bacteria. Especially, *Hungatella* and *Escherichia* were found predominantly in the comparison between DKD and DM and between DKD and healthy controls. This phenomenon has also been found consistently in the subgroup of clinically diagnosed DKD and biopsy-proven DN, indicating a potential pathogenic mechanism of *Hungatella* and *Escherichia* for DKD.

The genus *R. torques* was demonstrated to be inversely correlated with eGFR of DKD in this review. *R. torques* belongs to mucin-degrading bacteria, which has been suggested to be positively associated with insulin resistance and hyperglycemia (60, 61). The enrichment of *R. torques* was found to have harmful effect on the gut barrier function of elevated lipopolysaccharides translocation, leading to aggravated inflammation in type 2 diabetic rats (62), which might be associated with their renal injury effect.

The advantages of this review is that we systematically searched and screened eligible literature comparing gut microbiome between DKD and non-DKD participants, including diabetes, NDKD, and healthy volunteers. Additionally, all the fecal samples were analyzed using high-throughput sequencing, which may reduce the risk of bias from detection. A recent systematic review also focused on gut bacterial alteration in DKD (15), however, it only compared the differences of gut microbiota between DKD and healthy controls, and studies using bacterial culture and polymerase chain reaction for bacterial analysis were also included in the systematic review. Furthermore, patients with diabetes and other CKD were excluded, which also reduce the bias from participant selection.

Several deficiencies of this review should be considered. First, the definition of DKD was not consistent across the enrolled studies, including biopsy-proven DN and clinically diagnosed DKD, which lead to high heterogeneity of subject selection. Although we have carried out sensitivity analysis and

subgroup analysis and obtained relatively stable results, more homogeneous studies are still required to clarify the characteristics of gut microbiota in DKD. Second, some included studies had small sample sizes, while some studies did not match confounding factors between DKD and control group, such as age or sex, which may lead to potential bias. Third, not all of the studies reported the data of all specific outcomes, leading to limited available data, which may result in unstable results that do not fully reflect the underlying differences of gut microbiota. Fourth, most of the studies were conducted in China, and only one cohort was from Europe; therefore, it is still difficult to clarify the differences in intestinal microbiota of DKD patients between different ethnic groups.

Conclusions

In conclusion, this review indicated alterations of gut microbiota in DKD. Although there were no differences in α-diversity indices between DKD and DM, we found the enrichment of the genera *Hungatella*, *Bilophila*, and *Escherichia* in DKD group. A lower microbial richness and β-diversity were found in DKD compared to healthy controls, more specifically, the phylum *Actinobacteria*, and the genera *Hungatella*, *Butyricicoccus*, *Faecalibacterium*, and *Lachnospira* were proved to be the main differential bacteria. *Faecalibacterium* were significantly depleted in DKD compared to NDKD. Given the potential weakness, substantial heterogeneity, and limited available data, more high-quality evidence is needed to confirm the characteristics of gut microbiota in DKD.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization, YW. Methodology, SH, PC, ZZ, YL, MC, YX, and YW. Software, SH. Funding acquisition, SH and ZZ. All authors contributed to the manuscript writing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1018093/full#supplementary-material

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Stratification of diabetic kidney diseases *via* data-independent acquisition proteomics–based analysis of human kidney tissue specimens

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Aim: The aims of this study were to analyze the proteomic differences in renal tissues from patients with diabetes mellitus (DM) and diabetic kidney disease (DKD) and to select sensitive biomarkers for early identification of DKD progression.

Methods: Pressure cycling technology–pulse data-independent acquisition mass spectrometry was employed to investigate protein alterations in 36 formalin-fixed paraffin-embedded specimens. Then, bioinformatics analysis was performed to identify important signaling pathways and key molecules. Finally, the target proteins were validated in 60 blood and 30 urine samples.

Results: A total of 52 up- and 311 down-regulated differential proteins were identified as differing among the advanced DKD samples, early DKD samples, and DM controls (adjusted $p<0.05$). These differentially expressed proteins were mainly involved in ion transport, apoptosis regulation, and the inflammatory response. UniProt database analysis showed that these proteins were mostly enriched in signaling pathways related to metabolism, apoptosis, and inflammation. NBR1 was significantly up-regulated in both early and advanced DKD, with fold changes (FCs) of 175 and 184, respectively (both $p<0.01$). In addition, VPS37A and ATG4B were significantly down-regulated with DKD progression, with FCs of 0.140 and 0.088, respectively, in advanced DKD and 0.533 and 0.192, respectively, in early DKD compared with the DM control group (both $p<0.01$). Bioinformatics analysis showed that NBR1,

VPS37A, and ATG4B are closely related to autophagy. We also found that serum levels of the three proteins and urine levels of NBR1 decreased with disease progression. Moreover, there was a significant difference in serum VPS37A and ATG4B levels between patients with early and advanced DKD (both $p<0.05$). The immunohistochemistry assaay exhibited that the three proteins were expressed in renal tubular cells, and NBR1 was also expressed in the cystic wall of renal glomeruli.

Conclusion: The increase in NBR1 expression and the decrease in ATG4B and VPS37 expression in renal tissue are closely related to inhibition of the autophagy pathway, which may contribute to DKD development or progression. These three proteins may serve as sensitive serum biomarkers for early identification of DKD progression.

KEYWORDS

diabetic kidney disease, progression, autophagy, tissue proteomics, identification

Introduction

Approximately half of all patients with type 2 diabetes will develop diabetic kidney disease (DKD), which is clinically defined as the presence of impaired renal function, elevated urinary albumin excretion, or both (1). DKD is recognized as a leading cause of end-stage renal disease (2). In addition to causing increased mortality, DKD imposes severe health consequences and financial burdens on patients (3). The severity of DKD can be assessed by clinical and pathological methods, and DKD is pathologically graded into four stages (stage I to IV) according to the Renal Pathology Society classification system (4). Both stages 1 and 2 are described as early DKD, and stages 3 and 4 represent progression to advanced DKD (4, 5). In clinical practice, the urinary albumin excretion rate in 24 hours and the albumin-creatinine ratio are commonly used to diagnose DKD and monitor its progression. However, the microalbumin level in the urine is not a sensitive and specific predictor of DKD progression (6, 7). At present, there is a lack of sensitive indicators to predict and identify the progression of diabetic nephropathy, and finding new biomarkers to identify DKD in the early stages is a substantial challenge.

In past decades, proteomic approaches have been used in a number of biomarker studies. High-throughput profiling of the proteome is used to assess biological samples to identify, quantify, and discern the function of all observable proteins in health and disease (8). In the past 10 years, proteomic studies of DKD have enriched our knowledge of the molecular mechanisms involved in the pathogenesis of this condition (9). Using mass spectrometry (MS) techniques, many biomarkers in blood, urine, and tissue have been found that are valuable predictors of and diagnostic or prognostic biomarkers for DKD and its progression (10). Urinary CKD 273 score, serum C3f, MCP-1, transthyretin and cystatin C, and more are powerful predictors for DKD (10–13). However, many biomarker studies are limited by small sample sizes, heterogeneity of results, and a lack of large-scale validation studies. Due to the limitations of detection techniques and the difficulty in obtaining human kidney specimens, previous studies of the pathogenic mechanisms of DKD have been based on blood and urine samples from patients or animal models (14, 15). However, these approaches cannot fully clarify the actual molecular mechanisms of DKD, because they are not based on human renal tissue, which hinders the effort to find new sensitive biomarkers for the early identification of DKD progression.

Recently, the pressure cycling technique (PCT) was developed for use in semi-automatic assessment of small volumes of clinical tissue (16). In addition, Pulse-data-independent acquisition (DIA) technology, which is based on traditional DIA technology, has become available. PulseDIA divides a sample into multiple short gradient injections, each of which has a different mass spectral window, and the mass spectral data collected from the various injections are combined and analyzed to achieve a higher rate of peptide and protein identification than traditional DIA. PCT-PulseDIA is a combination of PCT and PulseDIA that provides higher quantitative accuracy and deeper proteomic coverage and is less time-consuming than traditional methods (17). These features make it suitable for proteomic analysis of kidney tissue from patients with DKD. Thus, in this study we subjected renal biopsy specimens from patients with DKD to proteomics analysis and validated the results by measuring

protein levels in blood and urine, with the aim of identifying effective biomarkers for the early diagnosis of DKD and identification of its progression.

Materials and methods

Renal tissue preparation

A total of 36 formalin-fixed paraffin-embedded (FFPE) kidney specimens were collected: four from patients with type 2 diabetes mellitus (DM control group), 17 from patients with early DKD (stage IIa-IIb), and 15 from patients with advanced DKD (stage III-IV). There were no significant differences in age, gender, BMI, or blood pressure among the three groups ($p>0.05$) (Table 1). Diagnoses were made by a single pathologist (Dr. JG Gong) according to Tervaert's pathological classification of diabetic nephropathy. The experiments were carried out with the understanding and written consent of each subject and in accordance with the declaration of Helsinki. The study was approved by the ethics committee of Zhejiang Provincial People's Hospital.

Pressure circulation technology–based sample preparation

The FFPE tissue samples were prepared for proteomic analysis as described previously (18). Samples were dewaxed, hydrated, and acidified using heptane, a decreasing ethanol series (100%, 90%, and 75%), and 0.1% formic acid in sequence. The samples were next kept under basic hydrolysis conditions in Tris-HCl (100 mM, pH=10) at 95°C for 30 min and then transferred to a solution containing 30 µL lysis buffer (6 M urea, 2 M thiourea), 5 µL Tris (2-carboxyethyl) phosphine (TECP, 10 mM), and 2.5 µL iodoacetamide (IAA) (40 mM). In PCT-Micro Tubes, the samples were lysed, reduced, and hydroxylated at 30°C using PCT (90 cycles, 45,000 psi, 30 s on-time and 10 s off-time). Trypsin (enzyme:substrate ratio, 1:50; Hualishi Scientific, China) and LysC (enzyme:substrate ratio, 1:40; Hualishi Scientific, China) were then added, followed by PCT-assisted digestion (120 cycles, 20,000 psi, 50 s on-time and 10 s off-time). Then, 1% trifluoroacetic acid (TFA) was added to terminate the digestion process. The resulting peptides were desalted with 2% acetonitrile (ACN) and 0.1% TFA and reconstituted. Peptide concentrations were measured with a Nanoscan (Analytic Jena, Germany) at A280, and samples were stored at 4°C for further analysis. All chemical reagents, unless otherwise specified, were obtained from Sigma-Aldrich.

PulseDIA proteomic analysis

PulseDIA MS was performed as previously described (19). The peptides from each sample were redissolved and analyzed on a nanoElute UHPLC (Bruker Daltonics, Germany) coupled to a timsTOF Pro mass spectrometer (Bruker Daltonics, Germany). Peptide powder was reconstituted in buffer A (0.1% formic acid in water). Peptide digests were separated at a flowrate of 300 nL/min using a 60 min gradient on a 15 cm analytical column with an integrated Toaster column oven at 50° C. The mobile phase B was 0.1% formic acid in ACN. The timsTOF Pro was operated in positive ion data-dependent acquisition Parallel Accumulation Serial Fragmentation (PASEF) mode. The capillary voltage was set to 4500 V. The MS and MS/MS spectra were acquired from 100 to 1,700 m/z and an ion mobility range (1/K0) from 0.7 to 1.3 Vs/cm^2. The ramp and accumulation time were set to 100 ms to achieve a duty cycle close to 100%. To perform diaPASEF acquisition, we defined two 15 Th isolation windows: from m/z 384 to 1008 and from m/z 475 to 1099. Spectronaut^{TM} (version 14.6) was used to compare all PulseDIA data against a renal-specific spectral library (20) including 539,631 peptide precursors, 448,338 peptides, 13,624 protein groups, and 9205 proteins with a false discovery rate of 0.01. The other parameters were set to the default values.

Collection and analysis of blood and urine samples

To validate the utility of the selected proteins, we recruited 150 patients with type 2 diabetes: 50 without DKD, 50 with early DKD, and 50 with advanced DKD. Venous blood and spot urine

TABLE 1 Biological characteristics of the patients in different groups.

Grouping	n	Age (year)	Sex (male, %)	BMI (kg/m2)	SBP (mmHg)	DBP (mmHg)
DM controls	4	58.00 ± 11.78	2/4 (50.00)	23.91 ± 4.46	134.00 ± 8.37	81.25 ± 7.32
Early DKD	17	55.53 ± 8.92	13/17 (76.47)	26.34 ± 4.05	142.75 ± 25.00	82.69 ± 15.14
Advanced DKD	15	56.00 ± 12.90	10/15 (66.67)	24.06 ± 2.32	150.67 ± 20.12	77.33 ± 12.92
p-value		0.53	0.61	0.19	0.20	0.40

†Data were presented by mean ± SD (for Age, BMI, SBP, and DBP) or percentage (for Sex). DM, diabetes mellitus; DKD, diabetic kidney disease; BMI: body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. P- value was tested by one way ANOVA.

were collected from 20 and 30 patients, respectively, from each group. Sera were separated from the blood samples by centrifugation at 1500 g for 5 minutes. Then, the levels of ATG4B, VPS37A, and NBR1 protein expression were measured by enzyme-linked immunosorbent assay (ELISA) (mlbio Co. Ltd., China) using a microplate reader (BIO-RAD, USA) to read the OD values of the reaction wells to calculate the concentrations. Urine creatinine levels were measured using a biochemical analyzer (AU5800, Beckman-Coulter, USA), and the protein:creatinine ratio was calculated to eliminate the influence of different urine volumes from each patient.

Immunohistochemistry assay of FFPE

To confirm the expression site of the three proteins in cells of renal tissue, an immunohistochemistry (IHC) assay was used. The detailed methods was as follows: 1) Deparaffinizing and rehydration: Immerse slides in xylene for 10 minutes, and repeat this step one time, then rehydrate two times by sequentially incubating with 100%, 95%, and 75% ethanol for 3 minutes each, finally rinse the slides with distilled water for 1 minute and place them in PBS buffer. 2) Antigen retrieval: Transfer slides to a microwave-proof container and cover with citrate buffer. After heating them in the microwave on medium power for 10 minutes, the slides were cooled in the citrate buffer for approximately 35 minutes. 3) Block endogenous peroxidase: Add appropriate amount of endogenous peroxidase blocker, and incubate at room temperature for 10 minutes;then rinse with PBS buffer for 3 minutes and 3 times. 4) Primary antibody incubation: Primary antibodies for NBR1 (polyclonal), VPS37A (monoclonal), and ATG4B (polyclonal) (proteintech, Wuhan Sanying, China) were diluted at 1:200, and 100 μ L of the antibodies was added for 60 min at 37°C, then the slides were rinsed with PBS buffer for 3 minutes and 3 times. 5) Enzyme-labeled antibody treatment: After adding 100 μ L of enzyme-labeled goat anti-rabbit IgG polymer solution (ZSGB-Bio, China),the slides were incubated at 37°C for 20 minutes and were rinsed with PBS buffer for 3 minutes and 3 times. 6) Color develops: Mix one drop of Liquid DAB plus chromogen immediately with 1 ml of substrate buffer to add on the slides, and incubate them at room temperature for 5 to 8 min. 7) Re-dying: Rinse the slides with tap water, and incubate with hematoxylin staining solution for 20 seconds. Then differentiate and rinse the slides to ensure the color returning to blue. 8) Dehydration and sealing: Immerse slides sequentially into 60%, 80%, 95% and 100% ethanol baths for 5 minutes each, then in xylene for 5 minutes. Repeat this step again in fresh xylene for 5 minutes. Mount the section with sufficient mounting media and cover with a cover slip, then air-dry them in a fume hood. 9) Results reading: The staining results were observed under a light microscope and read on the stained FFPE by a qualified pathologist.

Bioinformatics and statistical analyses

Statistically significant differences in protein expression in tissue samples, and their concentrations in serum and urine samples from the DM control group and patients with early DKD and advanced DKD, were determined by one-way analysis of variance (ANOVA), and p-values were adjusted using the Benjamini & Hochberg correction. P-values less than 0.05 were considered to be statistically significant. Soft clustering analysis of statistically significant differences in protein expression was performed using the R package "Mfuzz" (21). The average protein expression levels in each group were used as the input data for clustering. The time series were separated according to disease progression, with the initial stage being the DM controls. Metascape analysis was performed to outline the significant canonical pathways (22). The p-value was calculated in Metascape by right-tailed Fisher's exact test, and p-values less than 0.05 were considered significant.

Results

Patient characteristics and study design

In this study, we aimed to identify differentially expressed proteins in renal tissues and confirm them in blood and urine samples from patients with advanced DKD compared with patients with early DKD and DM controls. Advanced DKD and early DKD are defined as stage III/IV and stage IIa/IIb DKD, respectively, diagnosed using the Tervaert criteria for DKD pathological stages. Patients with type 2 diabetes without complications were included as the control group. For the first part of the study, we enrolled 36 patients with type 2 diabetes (T2M), including 4 with DM, 17 with early DKD (stage IIa-IIb), and 15 with advanced DKD (stage III-IV). The FFPE samples were successfully prepared, and the proteins were extracted by PCT. The DDA-MS data were then used to construct a tissue-specific spectral library of the FFPE tissues from the early DKD, advanced DKD, and control patients. All FFPE samples were subjected to PulseDIA to identify differentially expressed proteins. Finally, bioinformatics analysis was performed to determine the regulatory pathways that the differentially expressed proteins participate in. Samples for proteomic analysis were processed *via* a PCT-DIA workflow as described in the Methods section (Figure 1A). The histopathological characteristics are shown in Figure 1B. The clinical characteristics of the participants are shown in Table 1. For the second part of the study, 150 patients with T2D, including 50 DM controls, 50 patients with early DKD, and 50 patients with advanced DKD, were enrolled. There was no significant difference in gender, BMI, or blood pressure among the three groups (p>0.05), although there was a significant difference in

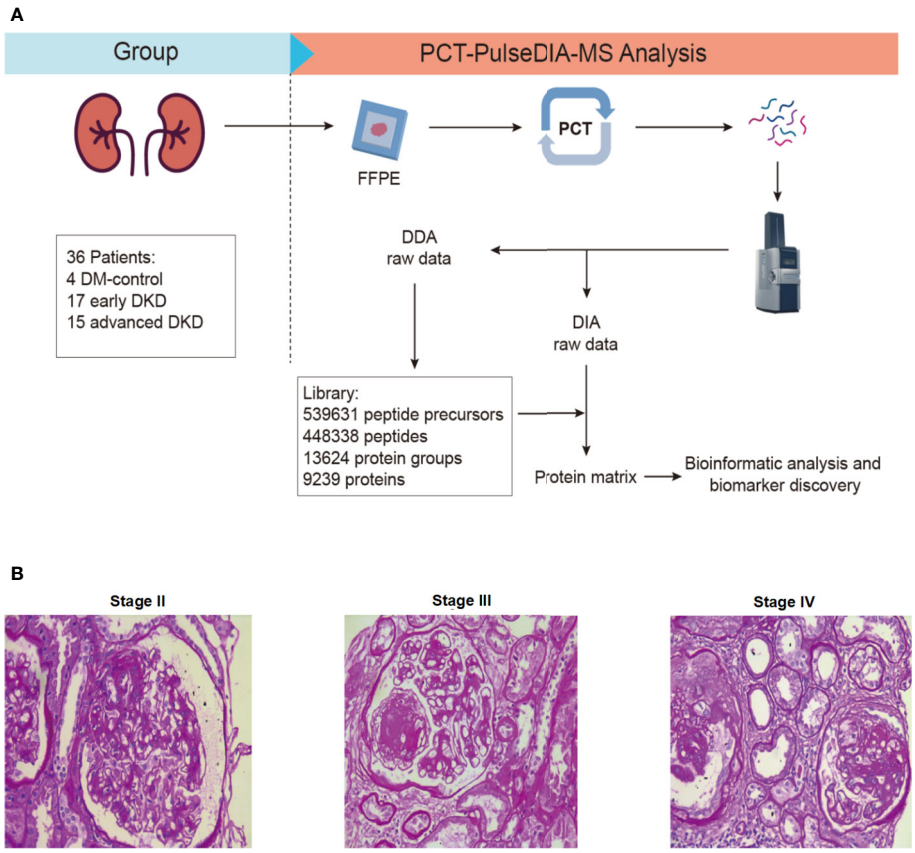

FIGURE 1
Study design. **(A)** FFPE-PCT-PulseDIA project design and workflow. **(B)** Histopathological characteristics of stage II, III, and IV DKD. FFPE: formalin-fixed paraffin-embedded; DKD: diabetic kidney disease.

age. The detailed clinical characteristics of these patients are not presented here. The expression levels of the proteins selected from the PCT-PulseDIA analysis were detected in 60 blood and 90 urine samples by ELISA to validate their utility as biomarkers.

Analysis of proteomics profiles

Two technical replicates of each sample in the discovery set were analyzed to enhance the robustness of the proteome maps generated from the FFPE tissues. In total, 36 specimens were analyzed by MS. We identified 9205 differentially expressed proteins in all the samples based on the proteomics data files. These proteins were related to DKD, and their expression levels are shown in the heatmap in Figure 2A. One-way ANOVA analysis comparing the three groups showed that the adjusted p-values for 502 of the proteins were less than 0.05. Of these, 52 were up-regulated and 311 were down-regulated with DKD progression, while the changes in expression of the rest of the proteins were irregular. The Venn diagram in Figure 2B shows the number of identified proteins displaying significant quantitative similarities and differences among the three groups. There were 8968, 9041, and 7432 proteins identified in the advanced DKD, early DKD, and DM control groups, respectively. A total of 7308 proteins were shared by all three groups, demonstrating that a large set of overlapping proteins (79.4%) was detected, which validated the robustness of the proteome maps to some extent. In addition, 88 and 131 proteins were only identified in advanced DKD and early DKD, respectively, while 50 proteins were only identified in the DM controls. Additionally, principal component analysis (PCA) of the 9205 differentially expressed proteins grouped by pathological stage (Figure 2C) revealed that the advanced DKD group shared more proteins with the early DKD group than with the DM control group.

Biological pathway analysis of proteins differentially expressed in DKD

The 502 proteins identified by clustering analysis as changing in expression level with disease progression are

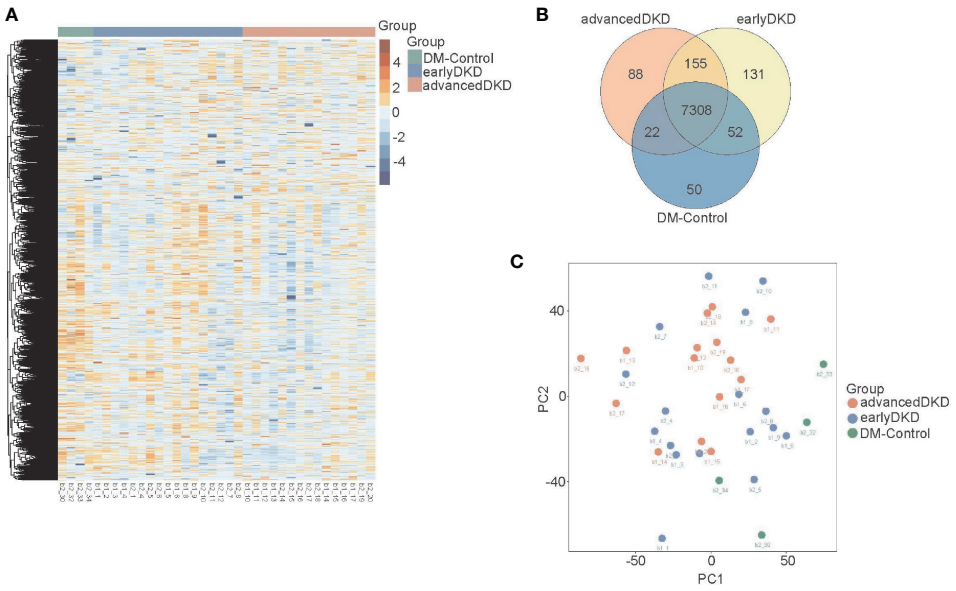

FIGURE 2
Renal tissue proteome profiles. **(A)** Heatmap showing 9205 protein that were expressed in the renal tissue of patients with DM control, early DKD, or advanced DKD. **(B)** Venn diagram showing overlapping protein expression among the three groups. **(C)** PCA of the 9205 proteins from the three groups. DM: diabetes mellitus; DKD: diabetic kidney disease. PCA: Principal component analysis.

shown in Figure 3A. Each group clustered proteins with different expression trends, namely up-regulation, down-regulation, up-regulation followed by down-regulation, or down-regulation followed by up-regulation. The results from the Mfuzz pathway analysis are shown in Figure S1. The proteins that were down-regulated with disease progression are mostly related to metabolism, cellular detoxification, and more, with cellular response to chemical stress and neutrophil degranulation appearing to be the most important pathways. The proteins that were down-regulated and then up-regulated are mainly involved in the regulation of proteolysis, protein phosphorylation, and more, with the main pathways being post-translational protein phosphorylation and neutrophil degranulation. The post-translational protein phosphorylation pathway was also highlighted in the analysis of proteins whose expression was first up-regulated and then down-regulated. In order to better analyze the differences between individual groups, Student's *t* test was used for pairwise group comparisons (fold change [FC]=1.50 was set as the cutoff value). Compared with the early DKD group, 138 and 173 proteins were up- and down-regulated in the advanced DKD group, respectively (Figure 3B); meanwhile, 389 and 376 up-regulated proteins and 1261 and 956 down-regulated proteins were found in the advanced DKD and early DKD groups, respectively, compared with DM-controls group (Figures 3C, D). Pathway analysis of the proteins identified in the variance analysis showed that the terminal complement pathway was the most significantly differentially regulated pathway between advanced DKD and early DKD, and that neutrophil degranulation and vesicle-mediated transport may play an important role in DKD development and progression (Figure S2).

Based on ANOVA and Mfuzz analysis with a cutoff value of an adjusted p-value of less than 0.05, 52 proteins showed an upward trend in expression level with DKD progression, while 311 proteins showed a downward trend. When the cutoff value was set to an adjusted p-value of less than 0.01, 17 proteins were up-regulated as DKD progressed, and 100 proteins were down-regulated. The heatmap in Figure 4A shows the expression levels of 363 proteins identified as being differentially expressed among the three groups. Then, PCA was performed by Mfuzz analysis (Figure S3). These proteins form a network that promotes the occurrence and progression of DKD (Figures 4B, C). Next, protein-protein interaction enrichment analysis was carried out using the following databases: STRING (23), BioGrid (24), OmniPath (25), and InWeb_IM (26). Only physical interactions identified by STRING (physical score >0.132) and BioGrid were used in the final analysis. The resulting network contains the subset of proteins that interact physically with at least one other protein on the list. If a protein-protein interaction network contains between 3 and 500 proteins, the Molecular Complex Detection (MCODE) algorithm (27) can be applied to identify

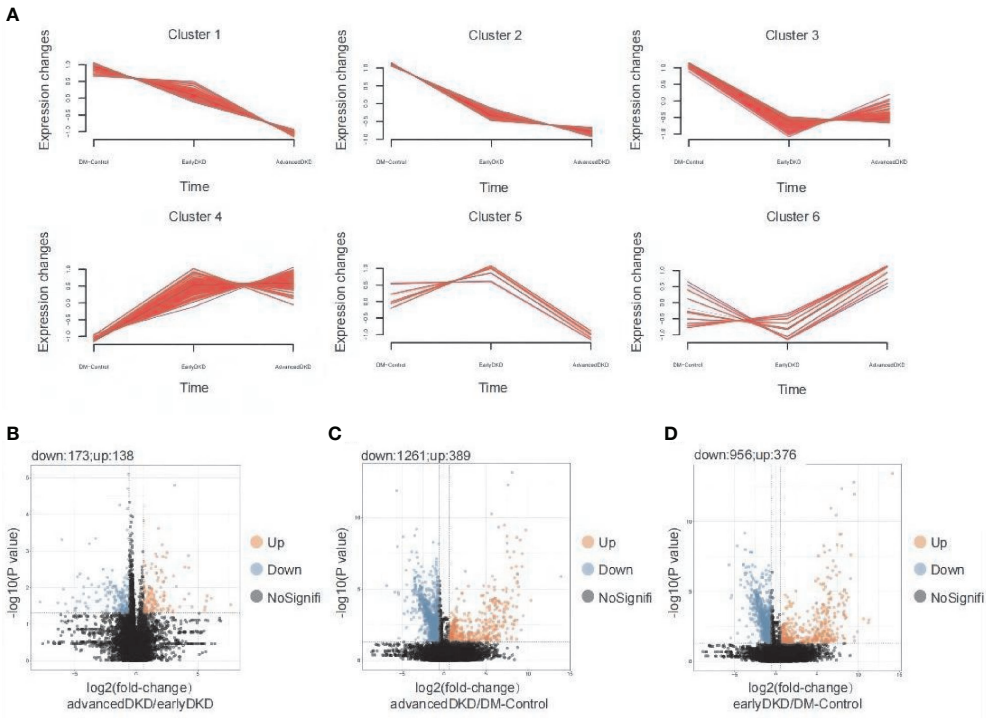

FIGURE 3
Detection and analysis of differentially expressed proteins. **(A)** Mfuzz analysis of 363 differentially expressed proteins. Volcano plot showing differentially expressed proteins between patients with advanced DKD vs. early DKD **(B)**, patients with advanced DKD vs. DM controls **(C)**, and patients with early DKD vs. DM controls **(D)** using a 1.5-fold–change cutoff and an adjusted p-value threshold of less than 0.05 by ANOVA (p adjusted by Benjamini & Hochberg correction). DM: diabetes mellitus; DKD: diabetic kidney disease; ANOVA: analysis of variance.

densely connected network components. The MCODE networks identified for individual protein lists generated in this study are shown in Figures 4B, C. Pathway and process enrichment analysis was applied to each MCODE component independently, and the three best-scoring terms by p-value were retained as the functional description of the corresponding components, as shown in the tables underneath the corresponding network plots in Figures 4B, C.

The enriched pathways identified by this analysis included multiple metabolic pathways, the iron death pathway, the autophagy pathway, the neutrophil threshing pathway, and other pathways involved in the occurrence and development of nephropathy. Annotation using the Metascape database identified cellular aminosyl metabolism, carbohydrate metabolism, proteolysis regulation, aminosaccharide and nucleotide glucose metabolism, cell REDOX homeostasis, and other biological metabolic processes as playing very important roles in this process, with adjusted p-values of less than 0.05 (Figure 4D) and 0.01 (Figure 4E). Three proteins in the autophagy pathway, ATG4B (Figure 5A), VPS37A (Figure 5B), and NBR1 (Figure 5C), showed significant changes in expression with progression of the disease. NBR1 was significantly up-regulated in both early and advanced DKD, with FCs of 175 and 184, respectively, compared to the DM control group (both $p<0.01$). Compared with the DM control group, VPS37A and ATG4B were significantly down-regulated with DKD progression, with FCs of 0.140 and 0.088 in advanced DKD, but 0.533 and 0.192 in early DKD, respectively (both $p<0.01$).

ELISA and immunohistochemistry analysis

ELISA analysis showed that serum levels of NBR1, VPS37A, and ATG4B decreased with disease progression. There were significant differences in NBR1 expression among the three groups [early DKD (67.67: 22.18-99.60) pg/ml vs. DM control (70.96: 35.73-205.57) pg/ml, vs. advanced DKD (35.94: 16.02-98.78) pg/ml; both $p<0.05$], and in VPS37A and ATG4B expression between the early and advanced DKD groups [(23.54: 11.29-35.24) and (27.49: 15.31-50.66) pg/ml vs. (15.31: 6.21-44.34) and (20.49: 8.97-59.18) pg/ml, $p<0.05$; respectively]. However, there was no statistical difference in NBR1 expression between the early and advanced DKD groups, or for VPS37A and ATG4B expression between the DM control and early DKD

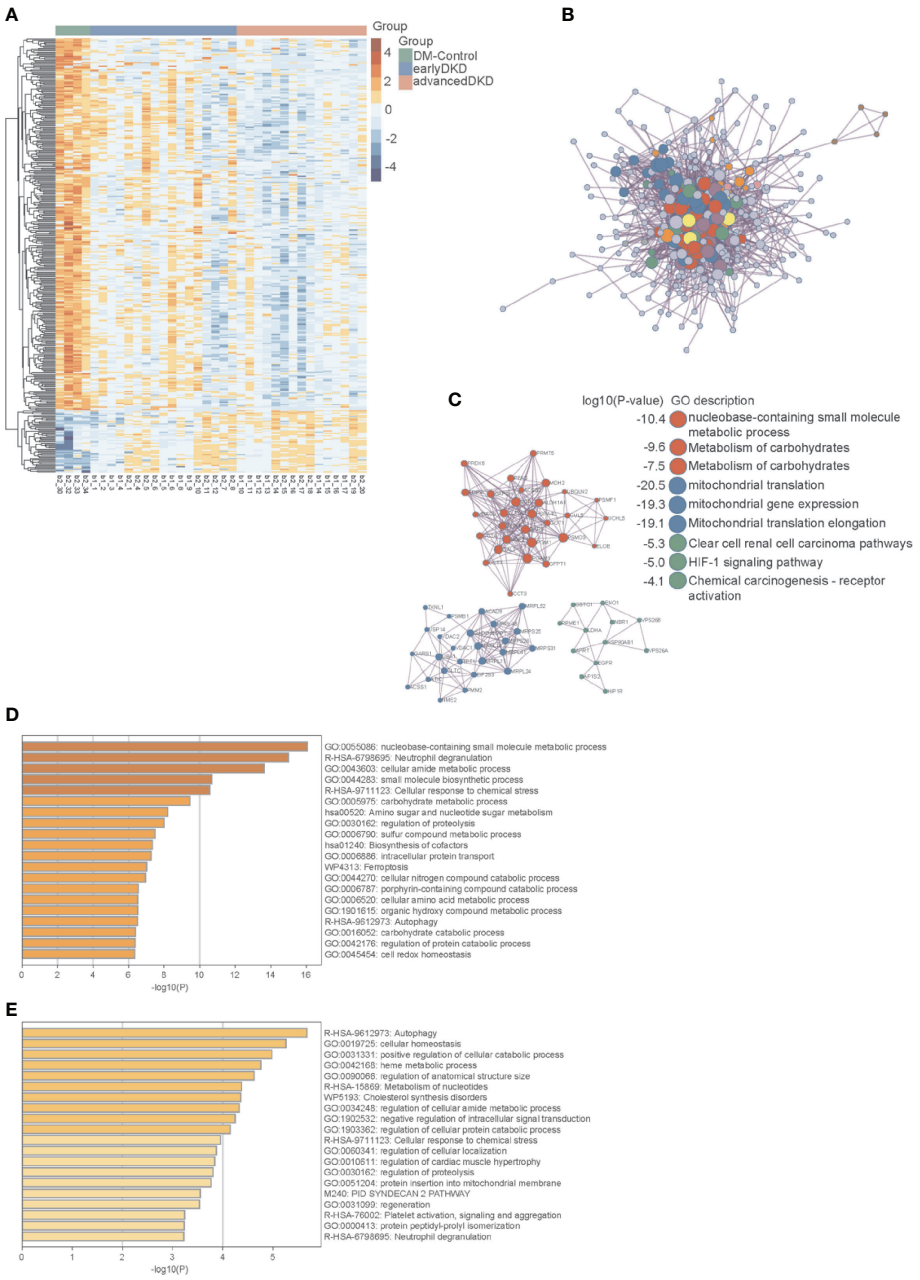

FIGURE 4
Protein interaction network and pathway enrichment analyses. **(A)** Heatmap showing the expression of 363 differentially expressed proteins in the DM control, early DKD, and advanced DKD groups. **(B)** Network interactions among major differentially expressed proteins. **(C)** MCODE components identified in the protein lists. **(D)** Pathways enriched in differentially expressed proteins using a cutoff value of p<0.05. **(E)** Pathways enriched in differentially expressed proteins using a cutoff value of p<0.01. The p-values were calculated and adjusted by one-way ANOVA followed by Benjamini & Hochberg correction. DM, diabetes mellitus; DKD, diabetic kidney disease; MCODE, Molecular Complex Detection.

groups (all p>0.05) (Figures 5D-F). Moreover, ELISA analysis of urine samples revealed that NBR1 exhibited remarkable differences in expression among the groups (p<0.05 respectively) (Figure 5G), but there were no statistically significant differences in VPS37A and ATG4B expression among the groups (all p>0.05) (Figures 5H, I). The FFPEs from the DM-controls were detected by IHC assay, respectively. The results of IHC assaay in each group exhibited that NBR1, VPS37A, and ATG4B were all expressed in the epithelial cells of renal tubule (Figures 6A-C), and NBR1 was also expressed in the cystic wall of renal glomeruli (Figures 6A1-3).

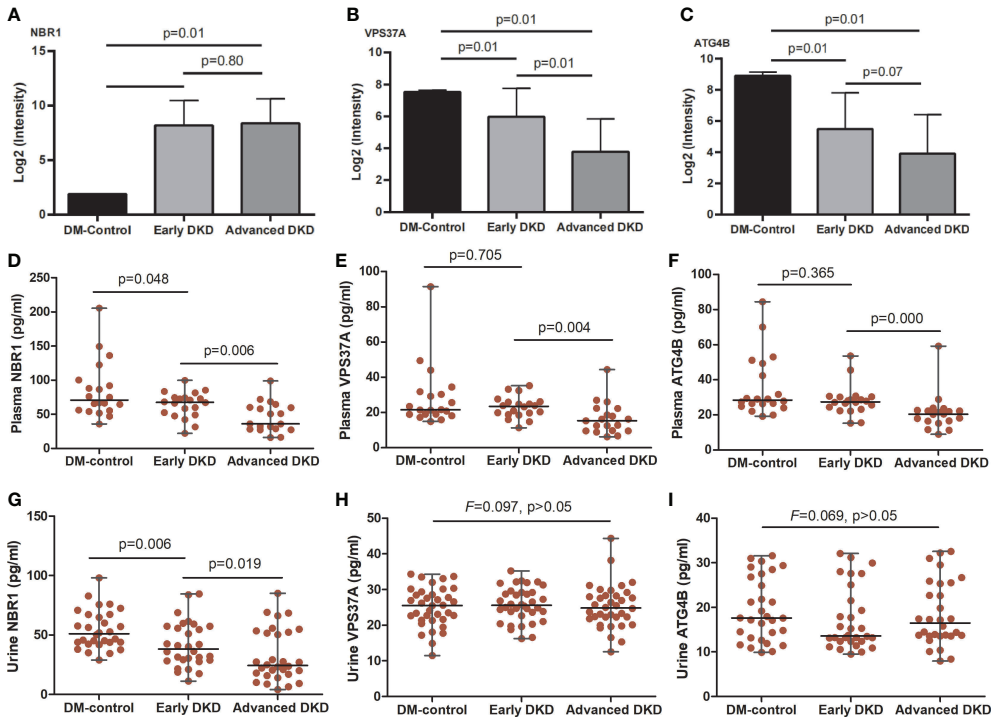

FIGURE 5
NBR1, VPS37A, and ATG4B expression in renal tissue, blood, and urine. **(A-C)** show the NBR1, VPS37A, and ATG4B expression, respectively, in renal tissue; the p-values were calculated by one-way ANOVA followed by *LSD*-test. **(D-F)** show serum levels and **(G-I)** show urine levels of NBR1, VPS37A, and ATG4B, respectively; the p-values were calculated by one-way ANOVA followed by Mann-Whitney *U*-test. ANOVA, analysis of variance.

Discussion

In the present study, by performing proteomics analysis of human kidney tissue and pathway annotation and enrichment, we found that a total of 363 proteins were differentially expressed among patients with advanced DKD, patients with early DKD, and DM controls, and that these proteins were mainly correlated with ion transport, apoptosis regulation, and the inflammatory response and enriched in signaling pathways related to metabolism, apoptosis, and inflammation. Moreover, we found that the autophagy-related protein NBR1 was significantly up-regulated in early and advanced DKD, but ATG4B and VPS37A were significantly down-regulated with DKD progression; indeed, the autophagy pathway ranked first out of the 20 main pathways that were most highly enriched in differentially regulated proteins. Finally, we found that NBR1, ATG4B, and VPS37A are mainly expressed in renal tubules, and that NBR1 levels in the sera and urine decreased with DKD progression. Our findings indicate that NBR1, ATG4B, and VPS37A may be new, sensitive biomarkers for early identification of DKD development or progression.

DKD can be diagnosed by clinical and pathological methods, and pathological diagnosis is the gold standard (4). However, early identification of DKD and monitoring its progression remain great challenges. In our study, we identified 52 proteins that were up-regulated and 311 proteins that were down-regulated with DKD progression. GO enrichment analysis showed that the differentially expressed proteins were involved in a variety of biological functions, including biological adhesion, biological regulation, developmental processes, localization, growth, immunity, response to stimuli, and prosodic processes, which is consistent with the previous studies of pathogenic factors (28). We also found that proteins that are differentially expressed among advanced DKD, early DKD, and DM are involved in multiple biological functions, including cell redox stability, small molecule biosynthesis, carbohydrates metabolism, and proteolysis regulation, which have also been mentioned in previous studies of the pathogenesis of DKD (29, 30).

Autophagy, which is an intracellular stress response, is currently of great interest to DKD researchers. In a diabetic state, hyperglycemia and hyperlipidemia in the kidney can inhibit autophagy, which leads to reduced autophagic activity in podocytes by blocking the AMP-activated protein kinase signaling pathway and activation of mammalian target of

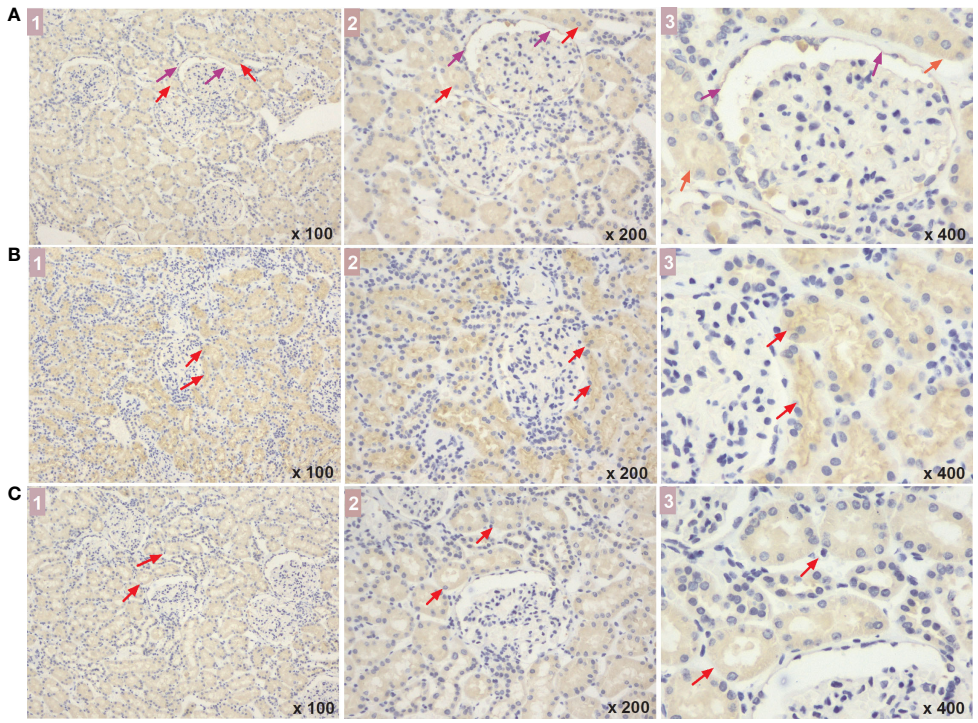

FIGURE 6
The expressed sites of NBR1, VPS37A, and ATG4B in renal tissue of DM patients. **(A-C)** show the NBR1, VPS37A, and ATG4B expression, respectively, and the marks of 1, 2, and 3 represent the different magnification time under a light microscope (x 100, x 200, and x 400, respectively). What marked with the red arrows means the expressions in renal tubular cells, and the expressions in the cystic wall of renal glomeruli was marked with the purple arrows. DM, diabetes mellitus.

rapamycin pathway (31). Recent studies have found that autophagy dysfunction can worsen renal hypertrophy, tubular damage, inflammation, fibrosis, and albuminuria in diabetic mice, indicating that autophagy plays a protective role in DKD (32). In our study, we found that multiple metabolic pathways, the iron death pathway, the autophagy pathway, the neutrophil threshing pathway, and other pathways are involved in the occurrence and development of nephropathy. The autophagy pathway ranked first out of the top 20 most significantly altered signaling pathways. We identified 12 differentially expressed proteins related to autophagy signaling, and NBR1 expression in particular was significantly increased in patients with DKD. NBR1 is closely related to renal cancer, and also participates in regulation of the autophagy pathway in renal clear cell cancer (33). Our results also showed that there was no obvious difference in NBR1 expression between patients with early and advanced DKD, suggesting that NBR1-mediated dysregulation of the autophagy pathway is likely to play a more important role in DKD development than in DKD progression. We further observed that ATG4B and VPS37A expression decreased significantly in kidney tissues from DM to advanced DKD, indicating the autophagy levels decreased along with DKD occurrence and progression.

In order to validate our results, we measured NBR1, ATG4B, and VPS37A levels in serum and urine samples from DM controls and patients with DKD. We found that the concentration of each of these biomarkers in serum decreased with disease progression. These results were the opposite of what was observed for NBR1 in the renal tissue analysis; further exploration is needed to determine the explanation for this observation. However, the changes in serum ATG4B and VPS37A levels were consistent with those seen in renal tissues, which partially validates the decrease in autophagy during DKD progression and suggests these two proteins could be used as blood biomarkers to evaluate DKD progression. Moreover, while we observed a similar trend in urine NBR1 levels throughout DKD development and progression, urine VPS37A and ATG4B levels did not vary significantly among the groups. Therefore, further prospective studies with a greater number of urine samples may be needed to obtain more definitive results. Moreover, we also used IHC assay to detect the expressed sites of the three proteins in renal cells of DM patients. We found that they were all expressed in the renal tubular epithelial cells, indicating that abnormal autophagy of renal tubule cells may be mainly contribute to the development and progression of DKD. However, NBR1 were also expressed in the cystic wall of renal glomeruli, indicating that its expression may be affected no matter whether the ball or the gym is

injured, so it is more likely to show the difference of blood and urine concentration expression in different stages.Therefore, a significant downward trend of this protein was found in the verification test of blood and urine, which further indicated that NBR1 may be an important autophagy protein associated with the development and progression of DKD. Although little is known about the role of ATG4B in DKD, miR-34a is thought to regulate autophagy by targeting ATG4B, leading to acute kidney injury (34). VPS37A is a very important protein in the cellular autophagy pathway, but its role in the pathogenesis of nephropathy is not well characterized. Its correlation with prostate cancer has been explored recently (35). VPS34 interacts with the PI3K complex to promote the nucleation of membrane bubbles and the formation of autophagosomes, and as such, plays a very important role in regulation of the autophagy pathway (36). However, confirming the correlation between VPS34 and nephropathy will require further intensive investigation. Therefore, the key proteins NBR1, ATG4B, and VPS37A, which have only rarely been reported to be associated with DKD in previous studies, are expected to serve as new, sensitive biomarkers for early identification of the development or progression of DKD.

The present study had some limitations. The difficulty of obtaining renal biopsy specimens resulted in a small sample number. The number of blood and urine samples used for the validation experiment was also relatively small. Therefore, the conclusions from our study need to be verified with further experiments in a larger sample size. Despite the size of our study, the results are relatively consistent with previous studies. Moreover, our study identified novel biomarkers associated with autophagy in DKD development or progression, and further confirmed the reliability of proteomics approaches.

Conclusions

In this study, we effectively identified several candidate markers of DKD progression by proteomic analysis of human kidney tissue. NBR1, ATG4B, and VPS37A, which are all members of the autophagy pathway, are expected to serve as effective biomarkers of DKD development and progression, and suggest that inhibition of autophagy may be a key event in DKD progression. This study may lead to improvement in the early identification of DKD development or progression and help identify new therapeutic targets for DKD.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Zhejiang Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

QHH and XHW designed the project. JGG revised the slides and collected the FFPE samples. ZXZ performed the experiments for discovery set and performed the targeted proteomic sample preparation and analysis. YC performed the Immunohistochemistry and ELISA ananlysis. QHH, XMF, XHW, and JRZ conducted the proteomic data analysis and document search. YWL designed and guided the additional experiments. QHH wrote the manuscript with inputs from all co-authors. XHW supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

ZZ was employed by Westlake Omics (Hangzhou) Biotechnology Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.995362/full#supplementary-material

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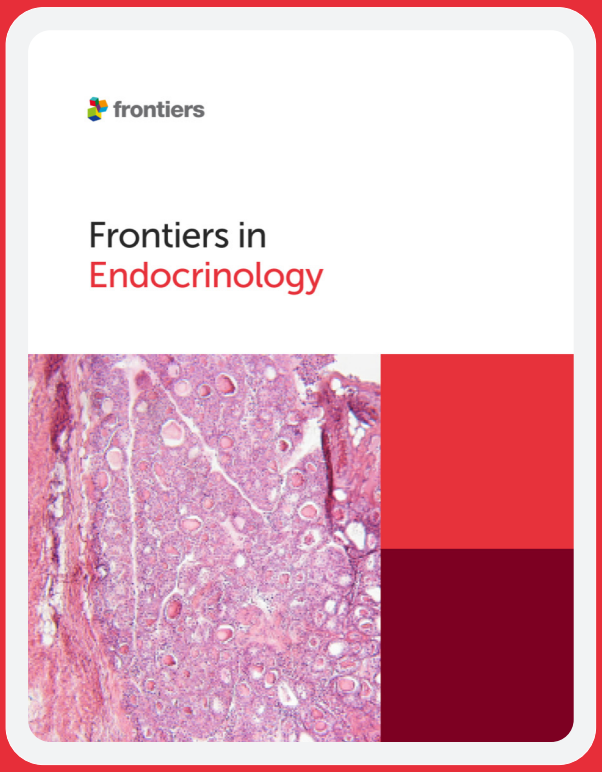

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