DIAGNOSIS, PREVENTION AND TREATMENT IN DIABETIC NEPHROPATHY

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DIAGNOSIS, PREVENTION AND TREATMENT IN DIABETIC NEPHROPATHY

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Editorial: Diagnosis, prevention and treatment in diabetic nephropathy

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

Diabetic nephropathy (DN) is one of the microvascular complications of diabetes affecting 30-40% of diabetic patients and represents the leading cause of end-stage kidney disease (ESKD). Treatment strategies are rare. Given the significant healthcare impact and high economic burden of DN, there is an urgent need for adequate and targeted management of the disease for early diagnosis and prevention of progression to ESKD.

Articles of this Research Topic provide a general overview of DN, highlight the importance of early detection of this disease, and suggest new diagnostic tools and treatment strategies.

The magnitude of the problem has been well described by Deng and colleagues, showing that diabetes-related chronic kidney disease (CKD) represents the sixth-leading cause of disability and fourth-leading cause of death globally Deng et al. In addition, they show that the middle socio-demographic index (SDI) quintile regions were the most interested by DN in 2019, while China, the United States and India were the countries with the highest burden of diabetes-related CKD Deng et al. Specifically, in a Bayesan age-period-cohort analysis, Wu et al. show that DN deaths in China could be on the rise, with DN deaths projected to reach 88803 in 2030, a 223.2% increase from 1990. Therefore, comprehensive prevention, early diagnosis and development of new therapeutic strategies are critical to reduce DN progression and related mortality.

DN is clinically characterized by proteinuria, hypertension, and progressive deterioration of renal function. Its main pathological features are mesangial expansion

to nodular accumulations, glomerular basement membrane thickening, glomerulosclerosis, tubular atrophy, interstitial inflammation and tubule-interstitial fibrosis.

Multiple factors are involved in the pathogenesis of DN, including a hyperglycemic environment, oxidative stress, inflammation, and fibrosis (Duan et al.). In particular, understanding the mechanisms underlying DN development is important to find new and specific biomarkers to make diagnosis, as shown in the review by Duan et al..

To date, glomerular injury is considered central to the pathogenesis of DN, and estimated glomerular filtration rate and albuminuria are, by far, the most commonly used parameters to assess renal function and DN progression. Recent evidence, however, focuses on the importance of renal tubular injury in determining reduced kidney function, even in the early stage of disease (Duan et al., Chang et al.). It has been shown that people with diabetes without proteinuria develop kidney disease (Chang et al.). Furthermore, in the absence of microalbuminuria, tubular plasma markers may also be associated with early renal injury (Duan et al.). Specifically, Duan et al. present in their review the latest evidences related to markers of renal tubular injury, including neutrophil gelatinase-associated lipocalin, kidney injury molecule 1, YKL-40, monocyte chemoattractant protein-1, cubilin and megalin. Another study by Lee et al. shows an association between tubulointerstitial injury in patients with DN and specific urinary tubulointerstitial mRNA biomarkers (LYZ, C3, FKBP5 and G6PC), and even assesses their predictive role in ESKD progression. This study, however, does not examine patients with early DN, so these results cannot be extrapolated to early kidney injury. The Research Topic also introduces other molecules as markers of renal damage. Indeed, in their study, Huang et al. show that higher urinary sodium excretion is associated with urinary albumin-to-creatinine ratio and DN risk, possibly through mechanisms dependent on vascular sclerosis or insulin resistance. No clear association between natriuresis and DN, however, has been found (Huang et al). Xu et al. analyze the role of specific lipid molecules in DN and find an association between lysophosphatidylethanolamine (LPE) and triacylglycerol (TAG) 54:2-FA18:1 and DN risk. Furthermore, they find that LPE, TAG 54:2-FA18:1 and phosphatidylethanolamine (PE) levels are associated with microalbuminuria (early DN) and macroalbuminuria (late DN), suggesting that these biomarkers can be used for early diagnosis of DN (Xu et al.). Han et al. present an innovative non-invasive diagnostic tool for diagnosing and predicting DN severity. Indeed, they assess the role of specific salivary glycopatterns in predicting DN progression. Another promising non-invasive diagnostic technique has been presented by Hu et al. in an animal model study that analyzes the role of magnetic resonance in the detection of preclinical DN, specifically through apparent diffusion coefficients and decreased fractional anisotropy techniques.

Several risk factors distinct from diabetes have been associated with the progression of DN, and some articles on this topic have been considered some of them, leading us to reflect on the importance of considering such components in DN prevention and possibly treatment.

In particular, a study by Yen et al. shows a strong correlation between diabetes and hypertension, suggesting that the coexistence of the two disorders is associated with a higher incidence of CKD. Furthermore, they show that diabetic patients who subsequently develop hypertension have a very high hazard ratio for end-stage-renal-disease compared with hypertensive patients who later develop diabetes. Along the same lines, in a metanalysis, Ren et al. show that patients with higher lipoprotein A levels have higher risk of developing DN. It is, however, unclear whether higher lipoprotein A levels are the result of abnormal renal metabolism due to loss kidney function or increased hepatic protein synthesis due to renal protein loss. Furthermore, it is unclear whether lipoprotein A may represent a marker of kidney injury or whether it is involved in the pathogenesis of DN through its atherogenic effects. CKD may also be exacerbated by acute kidney injury, which is a mortality risk factor for people with diabetes (Mo et al.). The effect of the gender has even been assessed in literature. In particular, multiple lines of evidence show an association between males and the risk of DN progression and death. Wang et al., however, are unable to find this association, and in a group of patients who underwent kidney biopsy, women have higher blood pressure, total cholesterol and LDL cholesterol compared with men, but a lower proportion of higher grades CKD histology.

Currently, clinical strategies to reduce DN progression are limited. Current treatment options include angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB). More recently, sodium-glucose cotransporter -2 (SGLT-2) inhibitors and novel non-steroidal mineral receptor antagonists have received attention for their anti-inflammatory and cardioprotective effects (Duan et al.). The meta-analysis by Li et al. describes the available evidence regarding the treatment of DN with tripterygium glycoside, a component of Chinese medicine with immunosuppressive effects. This work finds that tripterygium glycoside reduces serum and urinary biomarkers levels of DN progression, but at the risk of side effects. Another review by Wang et al. summarizes the results of various studies using mesenchymal stem cells and describes possible applications in DN therapy. More research, however, is needed to clarify the risks and benefits of these treatments (Li et al.).

Overall, all the articles of this Research Topic give a broad overview of new strategies for DN diagnosis, prevention and

treatment, providing new insights and future perspective for research in the field.

Author contributions

MR and FB wrote the article. MR, FB, MG and VT 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.

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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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Association Between Lipoprotein (A) and Diabetic Nephropathy in Patients With Type 2 Diabetes Mellitus: A Meta-Analysis

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Background: Lipoprotein (a) [Lp (a)] has been well recognized as a risk factor of cardiovascular disease. However, the association between serum Lp (a) and diabetic nephropathy in patients with type 2 diabetes mellitus (T2DM) remains unknown. We performed a meta-analysis to comprehensively evaluate the above association.

Methods: Observational studies aiming to evaluate the independent association between serum Lp (a) and diabetic nephropathy in T2DM patients were identified by systematic search of PubMed and Embase databases. A random-effect model which incorporated the potential intra-study heterogeneity was used for the meta-analysis.

Results: Eleven observational studies with 9304 T2DM patients were included. Results showed that compared to those with the lowest Lp (a), patients with the highest Lp (a) level had higher odds of diabetic nephropathy (adjusted odds ratio [OR]: 1.63, 95% confidence interval [CI]: 1.25–2.14, I^2 = 54%, P < 0.001). Meta-analysis of studies in which Lp (a) was presented as continuous variables showed consistent result (adjusted OR: 1.13 for 1 mg/dl increment of Lp (a), 95% CI: 1.03–1.24, I2 = 36%, P = 0.008). Subgroup analyses showed that study characteristics such as definitions of diabetic nephropathy and study design did not significantly affect the association (P for subgroup difference all > 0.05).

Conclusions: Higher serum Lp (a) in patients with T2DM is independently associated with higher odds of diabetic nephropathy. Large scale prospective cohort studies are needed to validate this finding. Moreover, the potential influence of Lp (a) lowering on renal function in T2DM patients may be further investigated.

Keywords: lipoprotein (a), type 2 diabetes mellitus, diabetic nephropathy, observational studies, meta-analysis

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INTRODUCTION

Patients with type 2 diabetes mellitus (T2DM) are vulnerable to kidney dysfunction, namely diabetic nephropathy (1). With the increasing incidence of DM globally, substantial patients with T2DM are suffering from diabetic nephropathy (2). As a common complication of T2DM, diabetic nephropathy has become one of the leading causes of end-stage renal disease (ESRD) all over the world (3). It has been reported that currently, over 20% of patients with diabetes ultimately develop

diabetic nephropathy, which has become a major cause of mortality in these patients (4). Although various risk factors for diabetic nephropathy have been proposed, such as age, race, duration of diabetes, hyperglycemia, dyslipidemia, and hypertension etc., further recognition of residual risk factors for diabetic nephropathy remain of clinical significance for the risk stratification and management of the disease (5, 6).

Lipoprotein (a) [Lp (a)] has been well recognized as a risk factor of cardiovascular disease due to its atherogenic effects (7, 8). Lp (a) is a low-density lipoprotein (LDL)-like particle consisting of an apolipoprotein B100 (Apo B) molecule linked to a very large glycoprotein known as apolipoprotein (a), or apo (a) (7). Accumulating evidence suggests that higher serum Lp (a) may be associated with impaired renal function in populations (9). Although early study has proposed that higher serum Lp (a) may also be associated with renal dysfunction in diabetic patients, subsequent studies evaluating the association between Lp (a) and diabetic nephropathy showed inconsistent results (10, 11). Some studies suggested that serum Lp (a) is related to higher odds of diabetic nephropathy in T2DM patients (12–17), while others did not (18–22). Therefore, relationship between Lp (a) level and diabetic nephropathy remains undetermined. Accordingly, in this study, we performed a meta-analysis of observational studies to comprehensively evaluate the association between Lp (a) and diabetic nephropathy in patients with T2DM.

METHODS

The meta-analysis was performed in accordance with the MOOSE (Meta-analysis of Observational Studies in Epidemiology) (23) and Cochrane's Handbook (24) guidelines.

Literature Search

Studies were identified *via* systematic search of electronic databases of PubMed and Embase *via* the following terms: (1) "Lp(a)" OR "Lp (a)" OR "lipoprotein(a)" OR "lipoprotein (a)"; (2) "diabetes" OR "diabetic"; and (3) "renal" OR "kidney" OR "nephropathy" OR "proteinuria" OR "albuminuria" OR "nephropathies". The search was limited to human studies published in English or Chinese. The reference lists of related original and review articles were also analyzed using a manual approach. The final literature search was performed on September 12, 2020.

Study Selection

The inclusion criteria for the studies were: (1) observational studies published as full-length articles; (2) included adult patients with T2DM; (3) evaluated the association between serum Lp (a) and diabetic nephropathy; and (4) reported the relative risk for this association after adjustment of potential confounding factors. Definitions of diabetic nephropathy were in accordance with the definitions applied among the included studies, which typically include the presence of microalbuminuria (urinary albumin-creatinine ratio [ACR]: 30–299 μg/mg) or macroalbuminuria (urinary ACR ≥ 300 μg/mg), and/or reduced renal function as presented by the reduced estimated glomerular infiltrating rate (eGFR) or elevated serum creatinine (SCr) (1). Reviews, editorials, preclinical studies, and studies irrelevant to the aim of current meta-analysis were excluded.

Data Extracting and Quality Evaluation

Literature search, data extraction, and quality assessment of the included studies were performed by two independent authors (XR and ZZ) according to the predefined inclusion criteria. Discrepancies were resolved by consensus. The extracted data included: (1) name of first author, publication year, and country where the study was performed; (2) study design characteristics; (3) participant characteristics, including health status, sample size, and sex; (4) patterns for Lp (a) analysis and cutoff values; (5) follow-up durations for cohort studies; (6) definitions of diabetic nephropathy; and (6) confounding factors adjusted in the multivariate analyses. The quality of each study was evaluated using the Newcastle-Ottawa Scale (25) which ranges from 1 to 9 stars and judges each study regarding three aspects: selection of the study groups; the comparability of the groups; and the ascertainment of the outcome of interest.

Statistical Analyses

We used odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) as the general measure for the association between Lp (a) and diabetic nephropathy in T2DM patients. For studies with Lp (a) analyzed as categorized variables, ORs of diabetic nephropathy in patients with the highest Lp (a) level compared to those with the lowest Lp (a) level were extracted. For studies with Lp (a) analyzed as continuous variables, ORs of diabetic nephropathy for each increment of 1mg/dl Lp (a) were extracted. Data of ORs and their corresponding stand errors (SEs) were calculated from 95% CIs or P values, and were logarithmically transformed to stabilize variance and normalized the distribution (24). For studies providing ORs with different adjusted factors, the ones with the most adequately adjusted factors were used in the meta-analysis. The Cochrane's Q test and estimation of I^2 statistic were used to evaluate the heterogeneity among the include cohort studies (26). A significant heterogeneity was considered if $I^2 > 50\%$. We used a random-effect model to synthesize the OR data because this model is considered as a more generalized method which incorporates the potential heterogeneity among the included studies (24). Sensitivity analyses, by omitting one individual study at a time, were performed to test the robustness of the results (27). Predefined subgroup analyses were performed to evaluate the influences of study characteristics on the outcome, including the definition of diabetic nephropathy, study design, and country of the study. The potential publication bias was assessed by visual inspection of the symmetry of the funnel plots. If more than 10 studies were included for each outcome, the Egger's regression asymmetry test was further performed for the evaluation of

potential publication bias (28). We used the RevMan (Version 5.1; Cochrane Collaboration, Oxford, UK) and STATA software (Version.12.0; Stata Corporation) for the meta-analysis and statistics.

RESULTS

Literature Search

The process of database search was summarized in **Figure 1**. Briefly, 882 articles were found *via* initial literature search of the PubMed and Embase databases after excluding of the duplications. Among them, 855 were excluded through screening of the titles and abstracts mainly because they were not relevant to the purpose of the meta-analysis. Subsequently, 27 potential relevant records underwent full-text review. Of these, 16 were further excluded for the reasons listed in **Figure 1**. Finally, eleven observational studies, including six prospective cohort studies (12, 15, 17, 19, 21, 22), three cross-sectional studies (13, 14, 18), and two nested case-control studies (16, 20), were included into the meta-analysis.

Study Characteristics and Quality Evaluation

The characteristics of the included studies were summarized in **Table 1**. Overall, eleven studies with 9304 T2DM patients were included. The studies were performed in Korea (12, 15), China (17, 18), Japan (14), Iran (16, 20), the United States (13, 19), Denmark (21), and the Netherlands (22). The mean ages of the patients varied from 53 to 69 years. The follow-up duration of the cohort studies varied from 2 to 11 years. In two studies, diabetic nephropathy was defined as the presence of microalbuminuria or macroalbuminuria (18, 22); in seven studies, diabetic nephropathy was defined as the decline of renal function as evidenced by reduced eGFR or elevated SCr (12, 13, 15–17, 19, 21); while in the other two studies, a combined outcome of albuminuria and/or decline of renal function was used (14, 20). Potential confounding factors, such as age, sex, smoking, body mass index, exercise, comorbidities, and use of antihypertensive medications, antidiabetic drugs, and lipid-lowering medications were adjusted to a varying degree. The NOS scores of the included studies ranged from seven to nine, indicating generally good study quality.

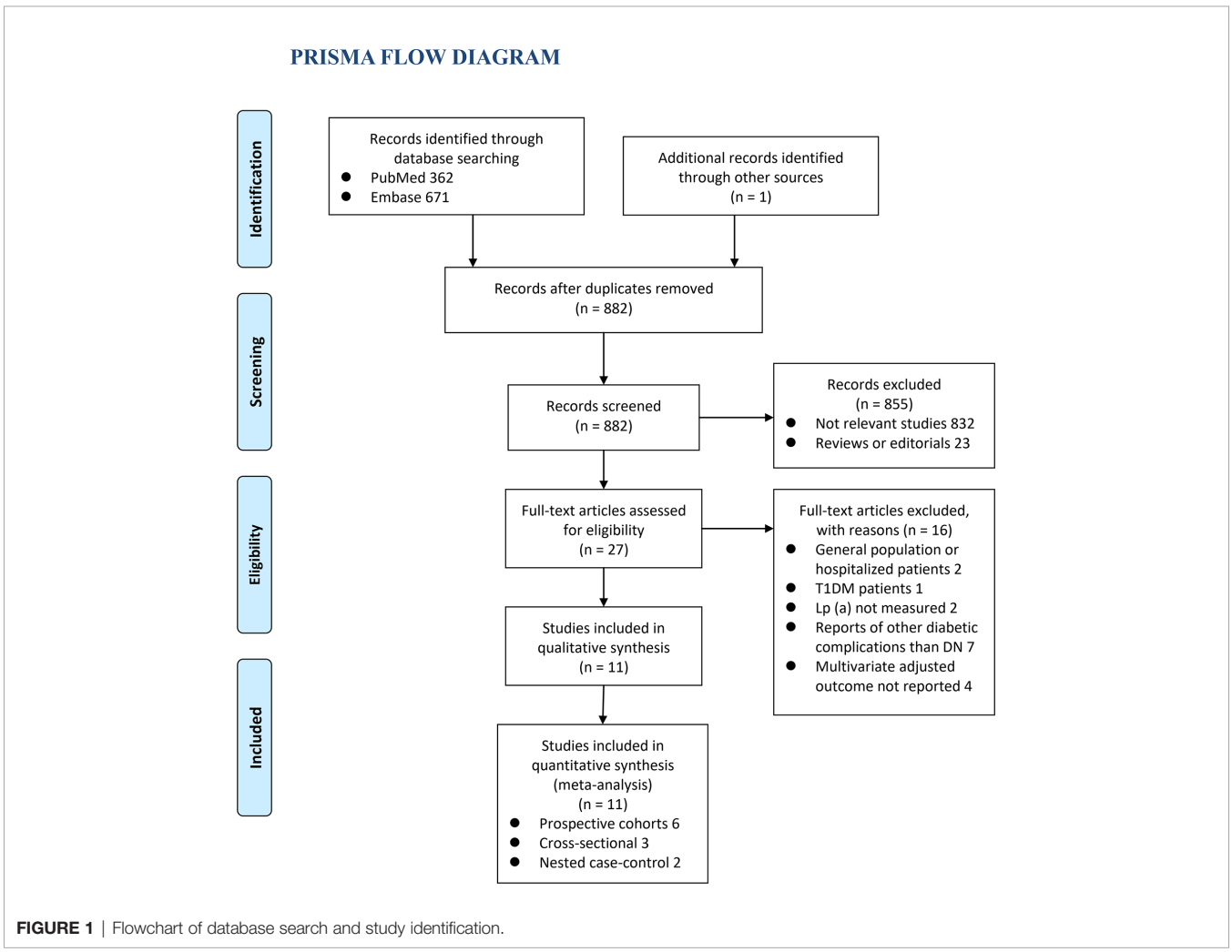

FIGURE 1 | Flowchart of database search and study identification.

TABLE 1 | Characteristics of the included observational studies.

Study	Country	Study design	Participants	Sample size	Mean age years	Male	Lp (a) presentation	Follow-up duration years	Definition of DN	Variables adjusted	Quality score
Song et al. (12)	Korea	PC	T2DM patients	81	59	44	Continuous	2	> 2-fold increase of follow-up SCr	Baseline SCr, SBP, and HbA1c	7
Tseng (18)	China	CS	T2DM patients	549	63	45	Continuous	NA	Microalbuminuria (ACR: 30–299 μg/mg) and macroalbuminuria (ACR ≥300 μg/mg)	Age, sex, BMI, diabetic duration, insulin use, SBP, use of statin/fibrate and use of ACEI/ARB	8
Lin et al. (19)	USA	PC	T2DM women	516	69	0	Q4 vs. Q1	11	eGFR decline of ≥ 25% during follow-up	Age, hypertension, BMI, ever smoked, physical activity, duration of T2DM, use of ACEI/ARB, baseline HbA1c and eGFR	9
Lin et al. (13)	USA	CS	T2DM patients without clinical CVD and with eGFR > 60 ml/min/1.73m^2	1852	59	64	Continuous, and ≥ 30 mg/dl vs. < 30mg/dl	NA	eGFR: 60–90 ml/min/1.73m^2	Age, sex, race, BMI, hypertension, lipid-lowering medications, HbA1c, HOMA-IR, duration on insulin, and urinary ACR	8
Yun et al. (15)	Korea	PC	T2DM patients with eGFR > 90 ml/min/1.73m^2	560	53	40	T3 vs. T1	10	eGFR < 60 ml/min/1.73m^2	Age, sex, diabetes duration, the presence of hypertension, CVD history, smoking, BMI, mean HbA1c, diabetic microvascular complication, FPG and Lp(a)-corrected LDL-C and medications like insulin, ACEI/ARB, statin, fenofibrate and acetylsalicylic acid	9
Senba et al. (14)	Japan	CS	T2DM patients	581	60	65	Above 90th percentile vs. below 30th percentile	NA	ACR ≥300 μg/mg and/or eGFR < 30 ml/min/1.73m^2	Age, sex, BMI, HbA1c, duration of T2DM, current drinking, current smoking, hypertension, dyslipidemia, CAD, and stroke	8
Aryan et al. (20)	Iran	NCC	T2DM patients	939	58	48	Continuous, and Q4 vs. Q1	NA	Microalbuminuria (ACR: 30–299 μg/mg), macroalbuminuria (ACR ≥300 μg/mg), or eGFR < 60 ml/min/1.73m^2	Age, sex, BMI, duration of diabetes, FPG, HbA1c, SBP, and the use of antihyperglycemic, antihypertensive and lipid-lowering medications	8
Singh et al. (22)	the Netherlands	PC	T2DM patients	1850	65	54	≥ 30 mg/dl vs. < 30mg/dl	7	Microalbuminuria (ACR: 30–299 μg/mg) and macroalbuminuria (ACR ≥300 μg/mg)	Age, sex, MAP, non-HDL-cholesterol, HDL-cholesterol, BMI, duration of type 2 diabetes, HbA1c and smoking	8
Heinrich et al. (21)	Denmark	PC	T2DM patients	198	59	74	Continuous	6	eGFR decline of ≥ 30% during follow-up	Age, sex, SBP, LDL-C, smoking, HbA1c, SCr and ACR	7
Xuan et al. (17)	China	PC	T2DM patients	1121	58	37	T3 vs. T1-2	4	eGFR < 60 ml/min/1.73m^2	Age, sex, BMI, FPG, SBP, TG, HDL-C, LDL-C, eGFR, smoking and drinking status, and use of antihypertensive drugs and antidiabetic drugs	9
Moosaie et al. (16)	Iran	NCC	T2DM patients	1057	57	47	Continuous, and ≥ 34 mg/dl vs. < 34 mg/dl	NA	eGFR < 44 ml/min/1.73m^2	Age, sex, SBP, HbA1c, BMI, use of anti-dyslipidemic drugs, eGFR, TG, LDL-C, HDL-C, non-HDL cholesterol, and waist/hip ratio	8

Lp (a), lipoprotein (a); DN, diabetic nephropathy; PC, prospective cohort; CS, cross-sectional; NCC, nested case-control; T2DM, type 2 diabetes mellitus; CVD, cardiovascular diseases; eGFR, estimated glomerular filtrating rate; Q, quartile; T, tertile; NA, not applicable; SCr, serum creatinine; ACR, albumin creatinine ratio; SBP, systolic blood pressure; HbA1c, glycated hemoglobulin; BMI, body mass index; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; HOMA-IR, homeostasis model assessment of insulin resistance; FPG, fasting plasma glucose; LDL-C, low-density lipoprotein cholesterol; CAD, coronary artery disease; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; MAP, mean arterial pressure.

Diabetic Nephropathy for Patients With Highest Versus Lowest Serum Lp (a) Levels

Eight studies (13–17, 19, 20, 22) evaluated the odds of diabetic nephropathy in T2DM patients with highest versus lowest serum Lp (a) levels. Pooled results with a random-effect model showed that patients with the highest Lp (a) level had higher odds of diabetic nephropathy (adjusted odds ratio [OR]: 1.63, 95% confidence interval [CI]: 1.25–2.14, I^2 = 54%, P <0.001; **Figure 2A**). Sensitivity analysis by omitting one study at a time did not significantly change the results (OR: 1.54–1.80, P all < 0.05). Subgroup analysis showed that definition of diabetic nephropathy or study design did not significantly affect the association (both P for subgroup difference >0.05; **Figures 2B** and **3A**). However, the association between Lp (a) and higher odds of diabetic nephropathy were mainly driven by studies which defined diabetic nephropathy as decline of renal function (five studies, pooled OR: 1.68, 95% CI: 1.26–2.44, P <0.001). Moreover, the association between Lp (a) and diabetic nephropathy seemed to be stronger in studies from Asia (OR: 2.29, 95% CI: 1.70–3.09, P < 0.001) than that in studies from non-Asia (OR: 1.24, 95% CI: 1.04–1.49, P = 0.02; P for subgroup difference < 0.001; **Figure 3B**).

Diabetic Nephropathy for the Increment of Serum Lp (a) of 1 mg/dl

Six studies (12, 13, 16, 18, 20, 21) evaluated the odds of diabetic nephropathy in T2DM patients with serum Lp (a) as continuous variables. Pooled results with a random-effect model showed that higher serum Lp (a) was associated with higher odds of diabetic nephropathy (adjusted OR: 1.13 for 1 mg/dl increment of Lp (a), 95% CI: 1.03–1.24, I^2 = 36%, P = 0.008; **Figure 4A**). Sensitivity analysis by omitting one study at a time did not significantly change the results (OR: 1.11–1.17, P all < 0.05). Subgroup analysis showed that definition of diabetic nephropathy, study design, or study country did not significantly affect the association (all P for subgroup difference > 0.05; **Figures 4B** and **5A, B**). Similarly, the association between Lp (a) and higher odds of diabetic nephropathy were mainly driven by studies which defined diabetic nephropathy as decline of renal function (four studies, pooled OR: 1.12, 95% CI: 1.03–1.21, P = 0.01).

Publication Bias

The funnel plots regarding the association between serum Lp (a) and diabetic nephropathy analyzed as categorized and

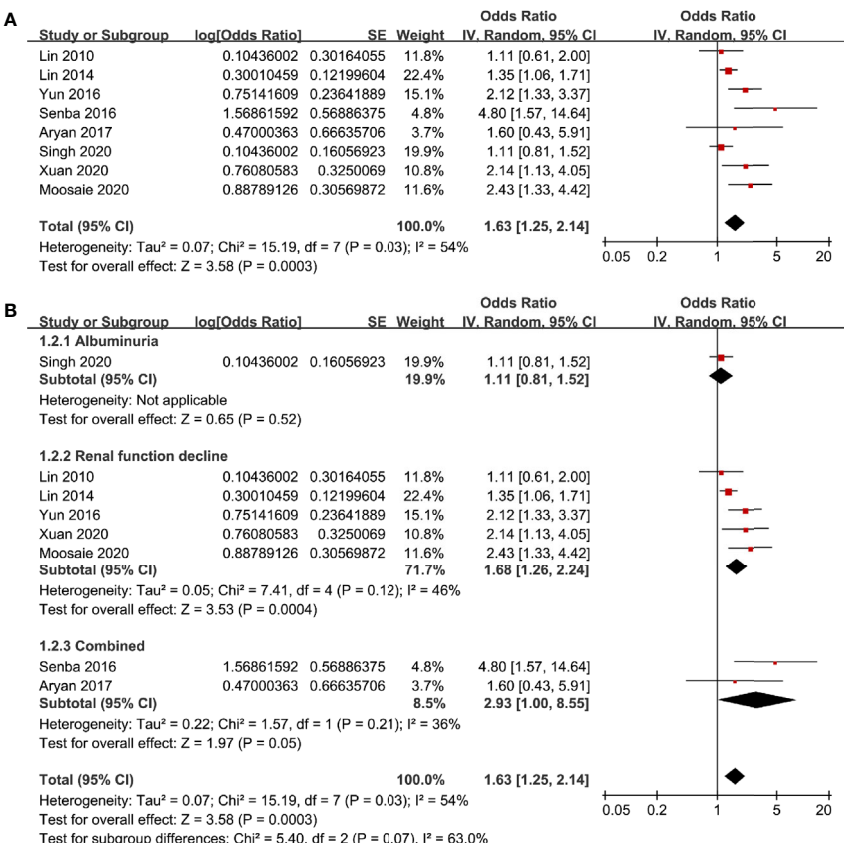

FIGURE 2 | Forest plots for the meta-analysis of the association between Lp (a) analyzed as categorized variables and diabetic nephropathy in T2DM patients; **(A)** results of main meta-analysis; and **(B)** results of subgroup analyses according to definition of diabetic nephropathy.

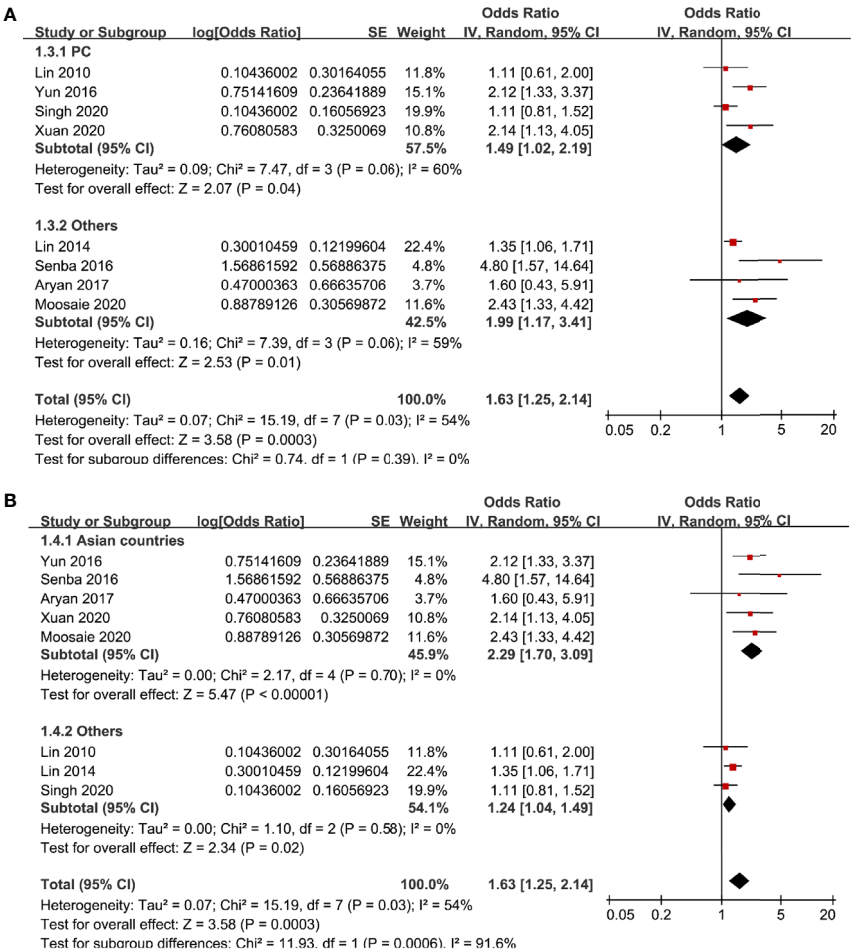

FIGURE 3 | Subgroup analyses for the association between Lp (a) analyzed as categorized variables and diabetic nephropathy in T2DM patients. **(A)** Subgroup analyses according to the study design and **(B)** subgroup analyses according to the study country.

continuous variables were shown in **Figures 6A, B**. The funnel plots were symmetry on visual inspection, suggesting low risk of publication bias. Egger's regression tests were not performed since less than 10 datasets were available for each meta-analysis.

DISCUSSION

This meta-analysis of observational studies showed that higher serum Lp (a) was associated with increased odds of diabetic nephropathy in patients with T2DM. The association between Lp (a) and diabetic nephropathy were consistent in studies with Lp (a) analyzed as categorized or continuous variables. Results of subgroup analyses suggested that the association between Lp (a) and diabetic nephropathy may not be significantly affected by the differences of the definitions of diabetic nephropathy or study design. However, the association between Lp (a) and diabetic nephropathy seemed to be stronger in studies from Asia than that in studies from non-Asia. Taken together, these results demonstrated that higher serum Lp (a) may be independently associated with higher odds of diabetic nephropathy in patients with T2DM. Although further large-scale prospective cohort studies are needed to confirm these findings, the potential influence of Lp (a) lowering on renal function in T2DM patients may be investigated in future studies.

To the best of our knowledge, this study is the first meta-analysis to summarize the relationship between serum Lp (a) and diabetic nephropathy in T2DM patients. The strengths of the meta-analysis may include the following. Firstly, the finding that Lp (a) is associated with diabetic nephropathy was based on multivariable adjusted data, indicating that the above association may be independent of potential confounding factors, such as age, sex, smoking, obesity, comorbidities, and concurrent medications. These results may suggest an independent association between Lp (a) and diabetic nephropathy. Secondly, studies with Lp (a) analyzed as categorized and continuous data were summarized separately and derived consistent results, which further validated the robustness of the meta-analysis. Thirdly, sensitivity analyses by omitting one study at a time did not significantly affect the results, suggesting the

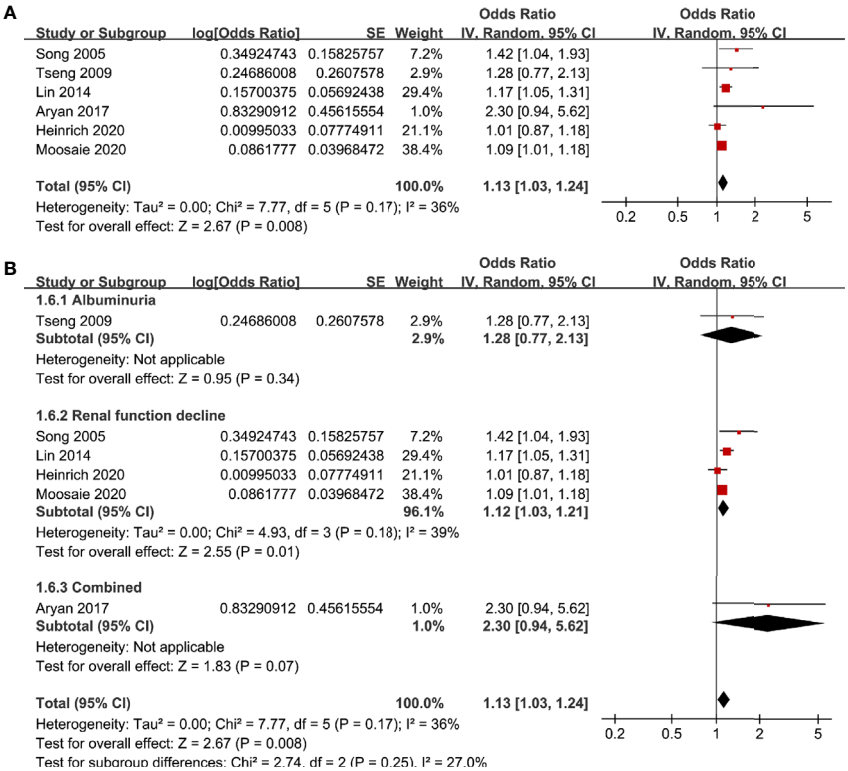

FIGURE 4 | Forest plots for the meta-analysis of the association between Lp (a) analyzed as continuous variables and diabetic nephropathy in T2DM patients. **(A)** Results of main meta-analysis and **(B)** results of subgroup analyses according to definition of diabetic nephropathy.

stability of the finding. Fourthly, multiple subgroup analyses were performed to evaluate the potential study characteristics on the association between Lp (a) and diabetic nephropathy. Although limited datasets were included for some stratum and interpretation of subgroup results should be cautiously, findings of subgroup analyses may be clinically relevant. Subgroup analysis did not show that differences in the definitions of diabetic nephropathy may significantly affect the results. However, the significant association between Lp (a) and diabetic nephropathy were mainly driven by studies with diabetic nephropathy defined as renal function decline. These findings suggests that higher Lp (a) may more likely to be associated with late changes of diabetic nephropathy evidenced by increased SCr or reduced eGFR (9). An early study in patients with chronic kidney disease (CKD) showed a rapid decrease of Lp (a) levels after renal transplantation, but not after initiation of hemodialysis, which suggested that the increase in Lp (a) seen in CKD is due to loss of functioning renal tissue (29). Moreover, a previous cohort study in T2DM patients without CKD at baseline also showed that baseline Lp (a) levels >30 mg/dl were associated with a decline in eGFR by 2.75 mL/min/year compared to 1.01 mL/min/year in subjects with baseline Lp(a) less than 30 mg/dl, which is consistent with our findings (30). In addition, we found that the association between Lp (a) and diabetic nephropathy seemed to be stronger in studies from Asia than that in studies from non-Asia. Previous studies have suggested the potential ethnic differences in the optimal cut-off values of Lp (a) (31) and its association with CVD risks (32, 33). Interestingly, an early cross-sectional study in the US population also showed that a low eGFR is associated with moderately greater Lp (a) levels in a race-ethnicity different manner (34). Future studies are needed to confirm whether an ethnic difference exists regarding the association between serum Lp (a) and diabetic nephropathy in T2DM patients.

The mechanisms underlying the potential association between Lp (a) and diabetic nephropathy may be multifactorial. The most likely explanation is that serum Lp (a) levels reflect a balance of Lp (a) synthesis in the liver and catabolism possibly involving kidney (9, 35). As previously mentioned, in patients with ESRD, Lp (a) levels were significantly increased compared to healthy controls, which were rapidly decreased after kidney transplantation but not after the initiation of hemolysis (29). These findings may support a metabolic role of the kidney in Lp (a) catabolism and suggest that the increase in Lp (a) seen in CKD is probably due to loss of functioning renal tissue (29). Besides, it has also been suggested that the increase in Lp (a) associated with protein-losing related renal disease is likely to be a result of a general increase in protein synthesis by the liver due to high urinary protein loss rather than decreased catabolism (36). Currently, it remains unclear whether increased Lp (a) plays key roles in the pathogenesis of diabetic nephropathy or it is just

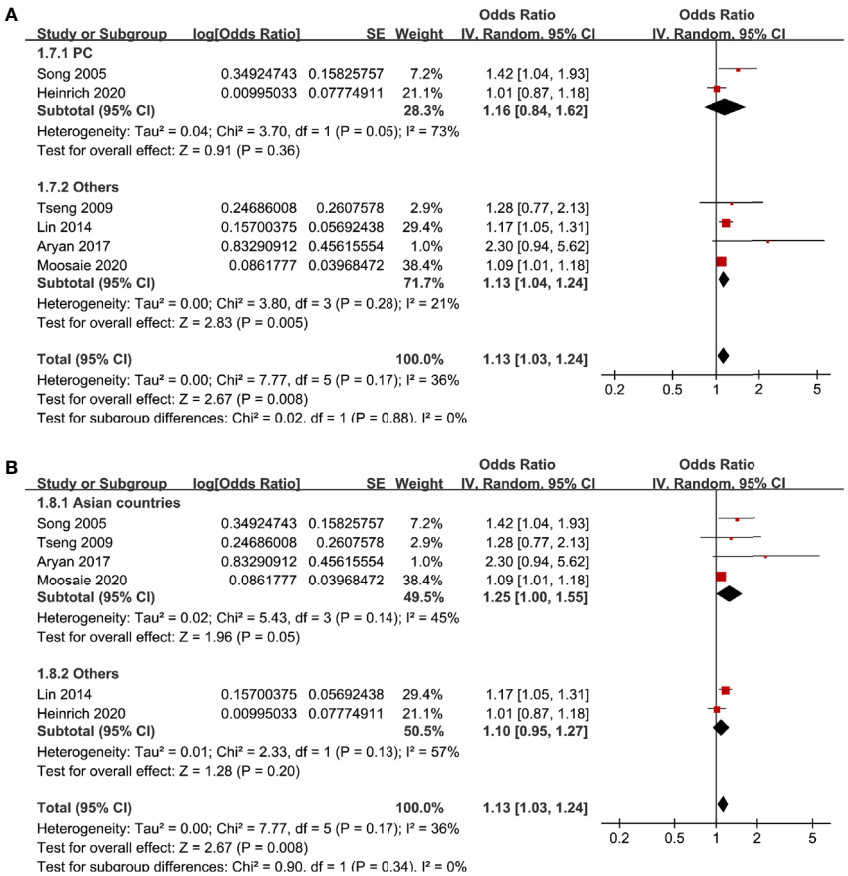

FIGURE 5 | Subgroup analyses for the association between Lp (a) analyzed as continuous variables and diabetic nephropathy in T2DM patients. **(A)** Subgroup analyses according to the study design and **(B)** subgroup analyses according to the study country.

a marker of impaired renal function. In view of the atherogenic role of Lp (a) and the importance of glomerular atherosclerosis in the pathogenesis of diabetic nephropathy (11), Lp (a) may be involved in the progression of diabetic nephropathy *via* its atherogenic effect. An early study *in vitro* study showed that low concentrations of Lp (a) stimulated growth of mesangial cells, whereas higher concentrations had antiproliferative or toxic effects, which may both have a negative impact on the course of renal disease (37). Another study showed that Lp (a) stimulated the growth of human mesangial cells and induced the activation of phospholipase C, which may therefore contribute to pathophysiology of renal disease (38). Moreover, oxidative stress has been confirmed to play a key role in the pathogenesis of diabetic renal complications (39, 40). Lp (a) was reported to induce the generation of oxygen-free radicals *in vitro*, which may partly contribute to kidney injury in diabetes (41). Besides, Lp(a) is susceptible to oxidative modification, leading to extensive formation of pro-inflammatory oxidized phospholipids, oxysterols, oxidized lipid-protein adducts in Lp(a) particles, which may perpetuate kidney injury (42). Future studies are needed to determine the possible pathophysiological mechanisms underlying the association between Lp (a) and diabetic nephropathy, and the potential influence of Lp (a) lowering on renal function in T2DM patients may also be investigated

Our study has limitations which should be considered when the results were interpreted. Firstly, considerable heterogeneity was detected among the included studies. Although we performed subgroup analysis to explore the potential influences of study characteristics such as definitions of diabetic nephropathy, study design, and study country, other factors may also contribute to the heterogeneity. Specifically, dietary natural products and medications that may affect serum Lp (a) are likely to modify the association between Lp (a) and diabetic nephropathy, such as phytosterol (43, 44), flaxseed (45), L-carnitine (46), and various lipid-lowering medications (47–49), which were rarely reported in the included studies. In addition, outcome of diabetic nephropathy should be optimally reported according to the stages of the disease (1). However, since various definitions of diabetic nephropathy was applied within the included studies, and none of them reported the outcome according to the stage of the disease, we were unable to determine the association between Lp(a) and diabetic nephropathy according to the disease stage. Besides, since the definitions of diabetic nephropathy within the included studies mainly focused on albuminuria and decreased eGFR, two key features of diabetic nephropathy (1), results of our study could reflection the

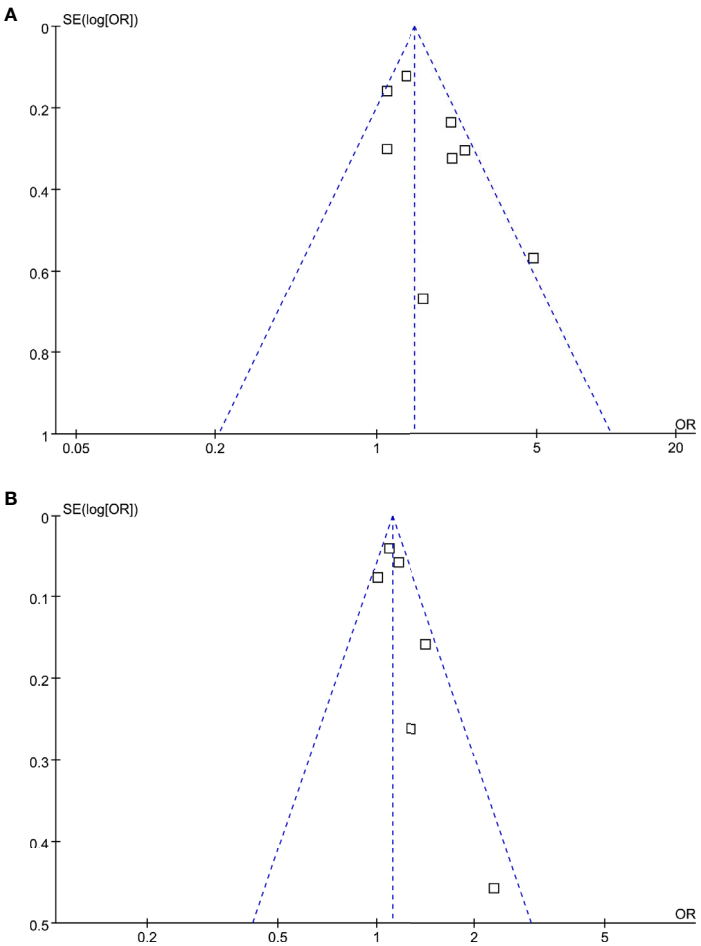

FIGURE 6 | Funnel plots for the publication bias underlying the meta-analysis of the association between Lp (a) and diabetic nephropathy in T2DM patients. **(A)** Forest plots for the meta-analysis of studies with Lp (a) analyzed as categorized variables and **(B)** forest plots for the meta-analysis of studies with Lp (a) analyzed as continuous variables.

association between Lp(a) and diabetic nephropathy. We have acknowledged this as a limitation of current meta-analysis, and future studies are needed to determine the association between Lp (a) and diabetic nephropathy of different disease stages. Moreover, since the individual patient data was not available, we could only perform subgroup analyses based on study-level data. In addition, in view of the limited datasets available for subgroup analyses, the results of subgroup analyses should be interpreted with caution. Furthermore, although we included studies with adjusted data, we could not exclude the existence of residual factors which may confound the association. Besides, it remains unknown whether the association is linear, or what the optimal cutoff value of Lp(a) is as for the prediction of diabetic nephropathy. Results of meta-analysis highlighted the importance of further studies (prospective cohort studies with large sample size) to investigate these issues. Finally, a causative association between higher serum Lp (a) and increased odds of diabetic nephropathy in T2DM patients should not be derived based on our finding since this study was a meta-analysis of observational studies.

In conclusion, higher serum Lp (a) in patients with T2DM is independently associated with higher odds of diabetic nephropathy. Large scale prospective cohort studies are needed to validate this finding, and the potential influence of Lp (a) lowering on renal function in T2DM patients may be further investigated.

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.

AUTHOR CONTRIBUTIONS

XR and ZY designed the study. XR and ZZ performed literature search, data extraction, and quality evaluation.

XR and ZY performed statistical analyses. XR wrote the manuscript. All authors reviewed and revised the manuscript, and approved the manuscript for submission. All authors contributed to the article and approved the submitted version.

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Efficacy and Safety of Tripterygium Glycoside in the Treatment of Diabetic Nephropathy: A Systematic Review and Meta-Analysis Based on the Duration of Medication

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Li Y, Miao R, Liu Y, Zhang J, Dou Z, Zhao L, Zhang Y, Huang Z, Xia Y and Han D (2021) Efficacy and Safety of Tripterygium Glycoside in the Treatment of Diabetic Nephropathy: A Systematic Review and Meta-Analysis Based on the Duration of Medication. Front. Endocrinol. 12:656621. doi: 10.3389/fendo.2021.656621

Aim: The aim of this study was to assess the clinical efficacy and safety of Tripterygium-derived glycosides (TG) after 3-month and 6-month of treatments of diabetic nephropathy (DN) and to resolve the conflict between medicine guidance and clinical practice for TG application.

Methods: We conducted a systematic review and meta-analysis of randomized controlled trials involving TG application in treating DN. We extensively searched PubMed, Cochrane Library, CNKI, VIP, Wan-Fang, CBM, Chinese Clinical Trial Registry, and WHO International Clinical Trial Registration Platform till November 2020, along with grey literature for diabetes and all other relevant publications to gather eligible studies. Based on the preset inclusion and exclusion criteria, document screening, quality assessment of methodology, and data extraction was conducted by two researchers independently. The methodological quality was assessed by the Cochrane risk test from the Cochrane Handbook 5.2, and then analyses were performed by Review Manager 5.3 (Rev Man 5.3). The quality of output evidence was classified by GRADE.

Results: Thirty-one eligible studies (2764 patients) were included for this meta-analysis. Our study results showed a comparable significant decrease in the 24 h-UTP and blood creatinine levels in DN patients from both 3-month and 6-month TG treatment groups, compared with the routine symptomatic treatment alone. To the contrary of the findings from the included studies, our results showed that the occurrence of serious adverse reaction events was significantly higher in the TG treated group with 6 months of treatment duration compared to that of 3 months of the treatment course. However, the total AR ratio was slightly varied while increasing the percent of severe adverse events. GRADE assessment indicated that the quality of evidence investigating TG-induced adverse reactions was moderate and that for 24 h-UTP and blood creatinine indicators were considerably low.

Conclusion: Combinatorial treatment regimen including TG can significantly decrease the pathological indicators for DN progression, while it can also simultaneously predispose the patient to a higher risk for developing severe adverse events, as the medicine guidance indicates. Notably, even in 3-month of course duration smaller percent of severe adverse events can get to a fatal high percent and is likely to increase proportionally as the TG treatment continues. This suggests that TG-mediated DN treatment duration should be optimized to even less than 3 continuous months to avoid adverse event onset-associated further medical complications in DN patients. In clinical practice, serious attention should be paid to these severe side-effects even in a course normally considered safe, and importantly more high-quality studies are urgently warranted to obtain detailed insights into the balance between the efficacy and safety profiles of TG application in treating DN.

Keywords: tripterygium glycosides, meta-analysis, systematic review, diabetic nephropathy, medication safety

INTRODUCTION

Diabetic nephropathy (DN) is characterized by degeneration of the renal microvasculature leading to leakage of proteins like albumin into the urine (commonly known as proteinuria), perturbed glomerular filtration, increased fluid retention, and high arterial blood pressure (1). DN frequently occurs in patients with diabetes mellitus, and its symptoms indicate chronic end-stage renal failure. DN is also considered as the leading cause of death in patients with chronic renal failure, with a prevalence rate of 4.8% (1, 2). Current therapeutic regimens include angiotensin II receptor blocker (ARB) and inhibitors for angiotensin-converting enzyme (ACE) to reduce the high blood pressure-associated renal complications and progression to DN (3, 4). Although some studies claim that ARBs are effective in treating proteinuria or albuminuria than ACE inhibitors, however, there are also contradictory results showing both of them have very similar efficacy in reducing proteinuria symptoms in primary hypertensive patients (5, 6). Despite the routine clinical applications of these drugs for slowing down DN progression, it has been challenging to reduce proteinuria completely in both diabetic- and non-diabetic patients with DN, particularly in cases of severe proteinuria. Furthermore, ARB/ACE inhibitors can induce fatal side-effects in patients with advanced stages of chronic renal insufficiency and having a serum creatinine level greater than 3mg/dL (7).

Hyper-activation of inflammatory responses has been frequently observed in patients with diabetes-related renal dysfunctions or chronic renal insufficiency (8), which further complicates the pathogenesis of DN (9–11). Recent investigations have greatly explored effective strategies for inhibition of renal infiltration of activated immune cells, cytokine storm, inflammatory responses, apoptosis, and podocyte injury as well to prevent DN progression (9, 12, 13). Notably, recently developed reno-protective drugs, such as sodium-glucose cotransporter-2 (SGLT-2) inhibitors, also have shown promising anti-inflammatory and anti-oxidative stress effects in the treatment of DN (14).

Tripterygium glycosides (TG) is an active compound found in the root extracts of *Tripterygium wilfordii* (TW). TG has been an essential component of traditional Chinese medicine for the treatment of glomerulonephritis and as a powerful immunosuppressive agent during kidney transplantation. TG has been increasingly utilized in the treatment of DN mainly due to its anti-inflammatory functions as well as its superb ability to prevent oxidation-induced membrane disruption in the glomerulus, thereby preventing DN progression and proteinuria (15–17). Moreover, combined therapy, including TG and ARB/ACE inhibitors, have been clinically applied as a potential therapeutic regimen against DN symptoms (18, 19).

In spite of multimodal pharmaceutical benefits of TG in treating several chronic life-threatening diseases, including DN and rheumatoid arthritis, TG-induced adverse events (AEs) have been mostly found to be systemic, organ-specific depending on the drug dose and duration of medication course (20). According to the drug overdose-related guidelines for TG tablets (Hunan Xieli Pharmaceutical Co. Ltd. National Medicine Standard Z43020138), long-term administration of TG may impact a number of physiological functions, e.g., digestion, blood pressure, renal and cardiovascular dysfunctions as TG-induced AEs, thus recommending a safe dose and course duration for 3 months. However, several other groups have shown no significant differences in safety profiles between 3 months and 6 months of treatment duration (21). Furthermore, recent studies investigating the impacts of different doses of TG in treating DN has revealed that 60 mg/day of TG is more effective than 30 mg/day for a period of 6 months with the same safety profile (22), and also TG is more efficient than valsartan in reducing the proteinuria level (23). The study also reports that there was no significant difference in AE occurrences between the placebo-treated and low-dose TG treated groups; however, in the double-dose group, only one patient exhibited an elevated level of alanine aminotransferase but was less than 2-fold compared to the baseline level, which was immediately normalized after symptomatic treatment.

Controversies exist between systematic reviews that focus on the clinical efficacy and adverse reactions of TG in the treatment

of DN. Some researchers believe that TG can improve some clinical indicators of patients with DN, such as 24 h-UTP and serum creatinine level (24–26), while others reached negative conclusions that TG can significantly induce adverse reactions in patients with DN (27, 28), and even questioned whether TG can be used to reduce the serum creatinine level of patients (28).

Thus, in this meta-analysis and systematic literature review regarding the safety and efficacy of TG administration in DN treatment and its relation to the duration of medication course, we have investigated the immediate necessities for "how" and "when" to balance between the standard medicine usage guidelines and empirical therapies for TG. This study will be highly beneficial to lay the theoretical foundations and practical clinical applications of TG to cure DN.

MATERIALS AND METHODS

Methods

This meta-analysis was conducted following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA). Prior ethics approval and consent of the participants were not required for this study since it involved only previously published RCT studies.

Literature Search Strategy

We performed a systematic search on PubMed, Cochrane Library, and WHO International Clinical Trial Registration Platform (ICTRP) for English language publications and China National Knowledge Internet (CNKI), China Science & Technology Journal Database (VIP), Wan-Fang digital periodical full-text database, Chinese Biomedical Literature Database (CBM) and Chinese Clinical Trial Registry (ChiCTR) for Chinese publications from database inception to November 05, 2020 based on the defined inclusion and exclusion criteria. The predefined English terms used for the search included "diabetic nephropathy" or "diabetic kidney disease" and "tripterygium glycosides" and terms related to randomized controlled trials (RCTs). In addition, we searched both manually and electronically for potentially eligible abstracts of newspapers, grey literature in the field of diabetes, along with any associated e-magazine references in order to prevent from missing any relevant studies. All the literature was published before November 2020. The detailed search strategy is provided in the supporting information.

Selection Criteria

Relevant studies were carefully screened by abstracts and titles, and then the eligibility criteria were applied based on PICOS as follows: (1) Patients: patients diagnosed with DN. The diagnostic criteria of DN was in accordance with 2007 National Kidney Foundation Kidney of Disease Outcomes Quality Initiative (NKF-K/DOQI): That is, in most patients with diabetes, the kidney damage should be considered as caused by diabetes if any of the following conditions occurs: massive proteinuria, diabetic retinopathy with microalbuminuria, microalbuminuria occurs in type 1 diabetes with the course of diabetes lasting for more than 10 years. (2) Intervention: TG combined with basic treatment applied, and the duration of TG treatment lasted for 3 or 6 months. (3) Comparison: TG combined with the basic treatment comparing with the basic treatment. (4) Outcomes: The efficacy of primary outcome was assessed by the changes in 24 h-urine total protein (24 h-UTP) and blood creatinine levels. (5) Study design: randomized controlled trials (RCTs) that applied TG in conventional treatment for DN for 3 months or 6 months of course duration, regardless of English or Chinese language, year of publication or country of publication.

Records retrieved from electronic searches were imported into reference management software (EndNote X7, Thomson Reuters, New York, NY, USA). After removing duplicate records, two reviewers independently screened the titles and abstracts of the remaining reports and subsequently investigated potentially eligible studies in full text. Inclusion and exclusion criteria are presented in **Table S1**. Differences in opinion between the two independent reviewers at any stage of the study processes were resolved by their mutual consensus or were further arbitrated by a third reviewer to reach a consensus.

Data Extraction and Risk-of-Bias Assessment

Two researchers extracted data independently, any discrepancies were discussed and resolved after consulted with a senior researcher. For each eligible trial, data on study characteristics, participants' baseline characteristics, key efficacy, and safety outcomes were extracted.

The risk of bias for the primary outcome was assessed by the respective tool developed by the Cochrane Collaboration 5.2. In this assessment, the following domains were considered: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other biases. The risk of bias for every domain was rated as high, unclear or low independently. Key domains included random sequence generation, allocation concealment, and incomplete outcome data. Publication bias was tested by funnel plot symmetry when at least 10 studies were available per meta-analysis.

Data Synthesis and Statistical Analysis

The extracted data were analyzed separately for 3-month treatment or 6-month treatment durations. We used the relative risk (RR) with 95% confidence intervals (CI) for dichotomous data, mean difference (MD) with 95% CI for continuous outcomes, heterogeneity quantified as high with I^2 values >50% and p<0.1. If substantial heterogeneity existed, a random-effects model was used to pool measures; otherwise, a fixed-effects model was used. Statistical analyses were performed using RevMan 5.3 (Nordic Cochrane Center, Copenhagen, Denmark, 2014).

RESULTS

Search Results and Study Characteristics

The study selection process is depicted in **Figure 1**. A total of 31 studies with 2764 participants were included after careful

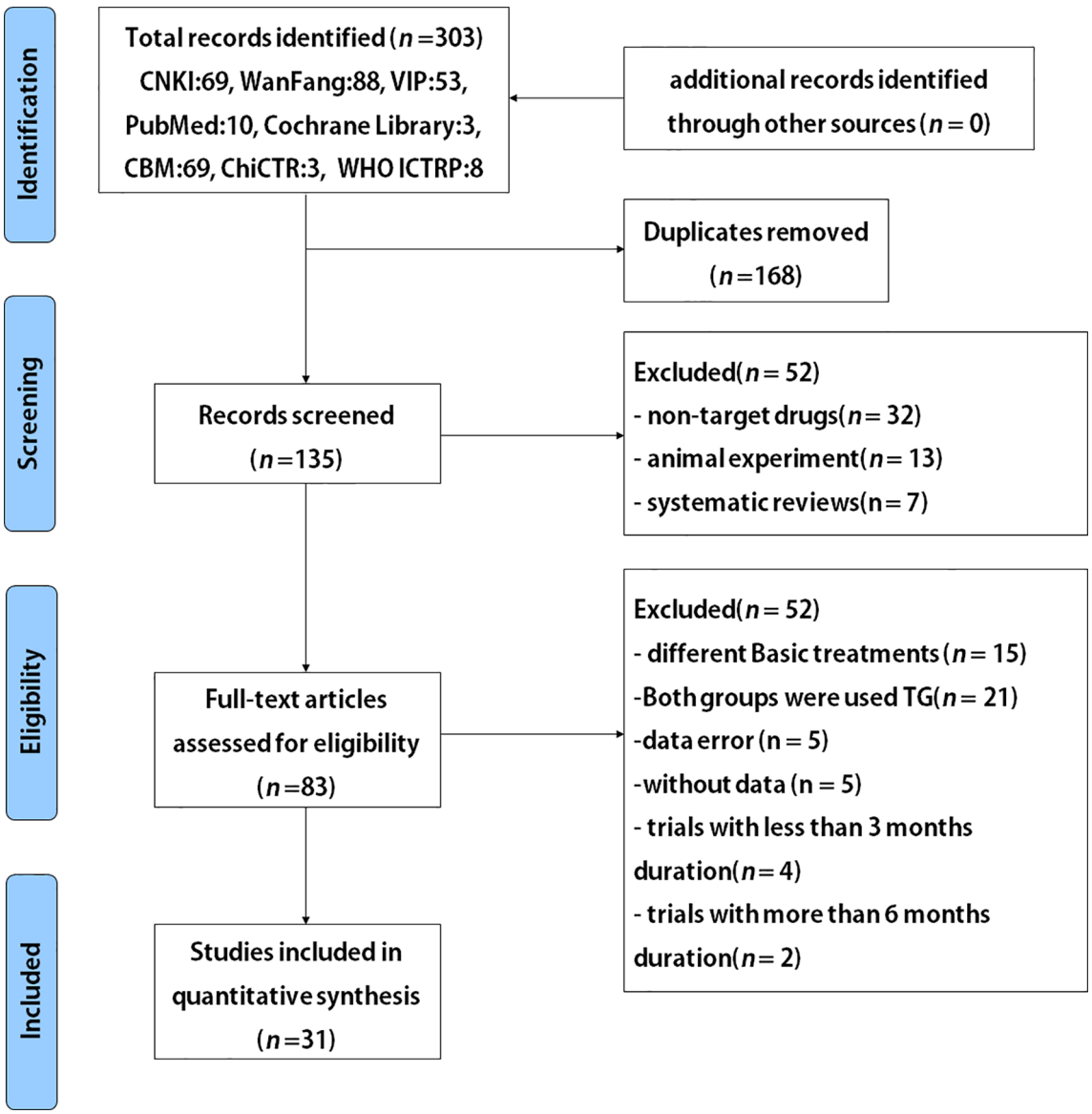

FIGURE 1 | Flowchart for randomized controlled trials (RCTs) to evaluate the use of TG after 3 and 6 months of treatment durations in patients with DN.

screening and evaluation for the systematic review (22, 29–58) (**Table 1**). All the studies were published in full-text, and participants' characteristics are shown in **Table 1**. The mean ages across the studies were ranged from 34.6 to 69.5 years, and 46.9% (1295) of the overall participants were women. Courses of treatment included were 3 months and 6 months. In all included trials TG combined with routine symptomatic treatments were compared with the symptomatic treatments alone. The symptomatic treatment targets included control of blood pressure and blood sugar, reduction of urinary protein content and blood creatinine level, etc. All studies exhibited that the baseline results for the TG-treated group and the corresponding control group were comparable.

Results With a Duration of 3-Month Treatment

Risk-of-Bias Assessment

Risk-of-bias assessment is shown in **Figure S1**. 24 h-UTP, blood creatinine, and AE outcome indicators were evaluated separately by the funnel plot, which revealed that no asymmetry existed in AEs, but significant publication bias was found for 24 h-UTP and blood creatinine (**Figures S2-4**).

24 h-UTP

Fourteen studies (29, 31–33, 38–47) including 1120 participants reported that the 24 h-UTP levels were significantly reduced [MD −0.30; 95% confidence interval (CI): −0.35 to −0.25;

TABLE 1 | Characteristics of studies and participants included in systematic review and meta-analysis.

Study, year [reference]	Number of participants	Dose of TG	Study duration, months	Outcomes of 24h-UTP and blood creatinine	Adverse event report
Li et al., 2012 (29)	128	60 mg/d	3	24h-UTP	Not reported
Lu et al. 2020 (30)	100	60 mg/d	3	Not reported	YES
Wang et al. 2013 (31)	64	-	3	24h-UTP, blood creatinine	YES
Gai et al. 2020 (32)	104	60 mg/d	3	24h-UTP, blood creatinine	No adverse event
Zhu et al. 2018 (33)	180	1mg/(kg·d)	3	24h-UTP, blood creatinine	YES
Wang et al., 2017 (34)	60	60 mg/d	3	Not reported	YES
Sun et al., 2019 (35)	100	1mg/(kg·d)	3	blood creatinine	YES
Yan et al., 2017 (36)	92	60 mg/d	3	Not reported	No adverse event
Liu et al., 2015 (37)	60	1mg/(kg·d)	3	blood creatinine	YES
Wang et al., 2018 (38)	80	60 mg/d	3	24h-UTP	No adverse event
Liu et al., 2015 (39)	40	1mg/(kg·d)	3	24h-UTP	YES
Zhang et al., 2015 (40)	40	1mg/(kg·d)	3	24h-UTP, blood creatinine	No adverse event
Shen et al., 2011 (41)	90	1mg/(kg·d)	3	24h-UTP, blood creatinine	YES
Shi et al., 2018 (42)	81	60 mg/d	3	24h-UTP, blood creatinine	YES
Sun et al., 2012 (43)	60	30-60 mg/d	3	24h-UTP, blood creatinine	YES
Shen et al., 2011 (44)	30	60 mg/d	3	24h-UTP	YES
Hao et al., 2017 (45)	58	120 mg/d	3	24h-UTP, blood creatinine	YES
Li et al., 2018 (46)	62	1-1.5 mg/(kg·d)	3	24h-UTP, blood creatinine	No adverse event
Ma et al., 2020 (47)	102	60 mg/d	3	24h-UTP, blood creatinine	YES
Wang et al.2018 (22)	40	60 mg/d	6	24h-UTP, blood creatinine	YES
Kong et al., 2013 (48)	60	60 mg/d	6	24h-UTP, blood creatinine	YES
Lu et al., 2019 (49)	200	30-60 mg/d	6	Not reported	YES
Yu et al., 2011 (50)	129	60 mg/d	6	24h-UTP, blood creatinine	YES
Yang et al., 2013 (51)	60	1-1.5 mg/(kg·d)	6	Not reported	YES
Li et al., 2020 (52)	80	10-60 mg/d	6	24h-UTP, blood creatinine	YES
Zhou et al., 2019 (53)	200	30-60 mg/d	6	24h-UTP, blood creatinine	Not reported
Xu et al., 2017 (54)	72	10-60 mg/d	6	blood creatinine	YES
Gao et al., 2012 (55)	80	60 mg/d	6	24h-UTP, blood creatinine	YES
Chen et al., 2009 (56)	119	1-2 mg/(kg·d)	6	24h-UTP, blood creatinine	YES
Shan et al., 2013 (57)	70	1mg/(kg·d)	6	Not reported	YES
Zhou et al., 2019 (58)	122	10-60 mg/d	6	Not reported	YES

I^2 = 98%] after 3-month of combined TG treatment compared with the non-TG regular treatment alone (**Figure 2**).

A subgroup analysis was conducted to reduce the heterogeneity in the results. The studies were divided into 4 subgroups (group 1: range >3 g, group 2: range 2.8-3 g, group 3: range 1.8-2.8 g, group 4: range 0.2-1.5 g) according to the baseline level of 24 h-UTP. The results showed that heterogeneity was reduced in the first three subgroups (**Figure 3**), suggesting that the difference in the 24 h-UTP baseline level was one of the critical heterogeneity sources. However, group 4 still had significant heterogeneity (I^2 = 98%), which might be due to the limited number of available full-text literatures that could not be further categorized, resulting in the substantial variation at the baseline level of group 4 than other groups.

Blood Creatinine

12 studies (31–33, 35, 37, 40–43, 45–47) including 1002 participants reported that the blood creatinine levels were significantly decreased [MD – 12.63; 95% CI: -21.96 to -3.31; I^2 = 98%] after 3-month of combined TG treatment compared with the non-TG regular treatment alone. (**Figure 4**).

A similar subgroup analysis was conducted to reduce the heterogeneity in the results. Likewise, studies were divided into 3 subgroups according to the baseline level of blood creatinine (group 1: range 160-200 μmol/L, group 2: range 99-130μmol/L, group 3: range 60-92μmol/L). In the first two subgroups, the heterogeneity was reduced to an acceptable degree, but in the third subgroup, high heterogeneity was still existed (**Figure 5**). A *post hoc* sensitivity analysis was conducted to explore the heterogeneity origin in the third subgroup results. Notably, exclusion of a trial reported by Li (2018) (46)reduced the heterogeneity to a comparable level with respect to other subgroups [MD in blood creatinine -1.56; 95% CI: -2.92 to – 0.20; I^2 = 0%], suggesting that the difference in the blood creatinine baseline level of the patients was the main source of heterogeneity in this meta-analysis.

Adverse Reactions

Adverse reaction events were reported in 13 studies (29, 30, 32–34, 36, 38, 40–44, 47), including 1148 subjects after 3-month of combined treatment with TG, which significantly increased the adverse reaction events [MD 2.02; 95% CI: 1.35 to 3.00; I^2 = 0%], compared with non-TG regular treatments (**Figure 6**). This data was inconsistent with the findings of the included clinical studies involving TG administration that TG could not induce significant AEs even after 3 months of continuous treatment duration.

In addition, the AEs related to the treatments combined with TG mainly reflected symptoms of leukopenia and abnormal liver functions (**Table 2**).

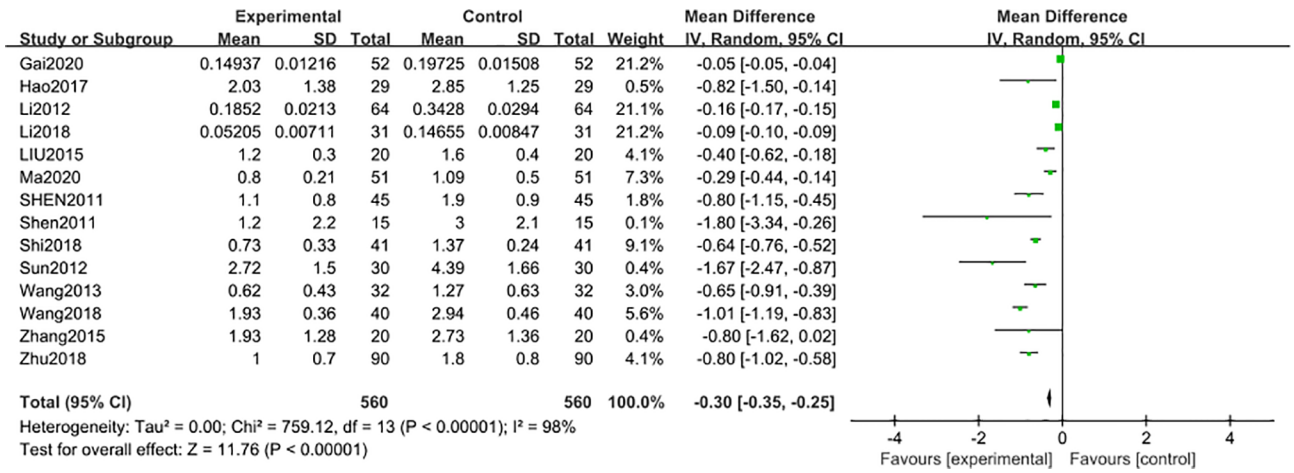

FIGURE 2 | 24 h-UTP after 3 months: comparison of treatment combined with TG against basic treatment.

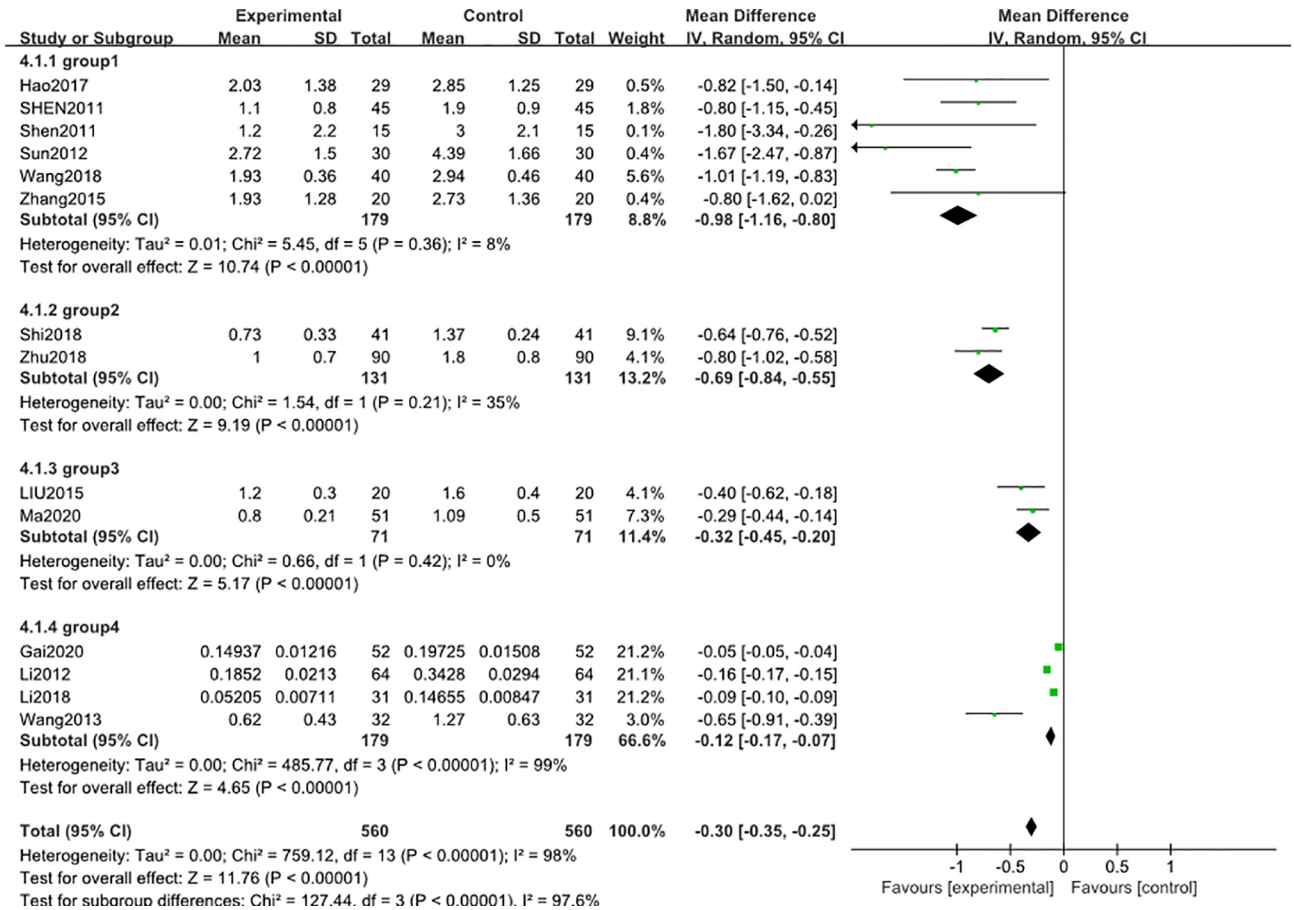

FIGURE 3 | Subgroup analysis of 24 h-UTP level after 3 months: comparison of treatment combined with TG against basic treatment (group 1: range 3 g-, group 2: range 2.8-3 g, group 3: range 1.8-2.8 g, & group 4: range 0.2-1.5 g).

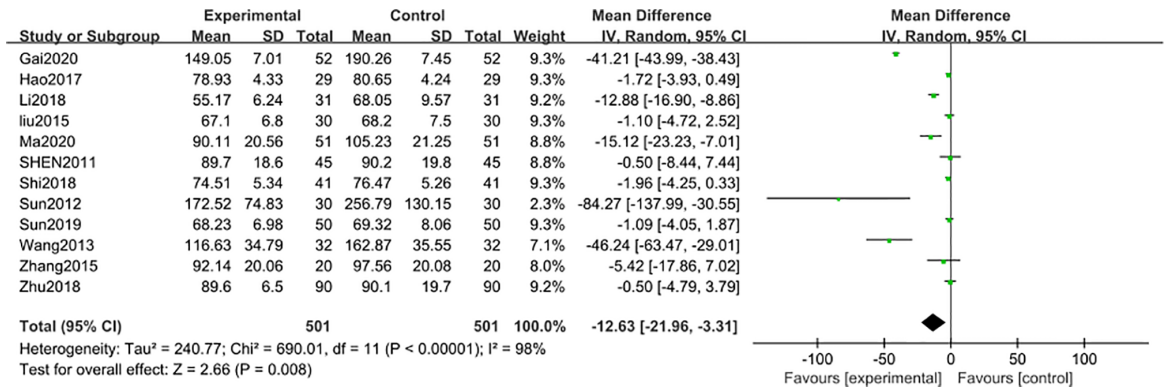

FIGURE 4 | Blood creatinine level after 3 months: comparison of treatment combined with TG against basic treatment.

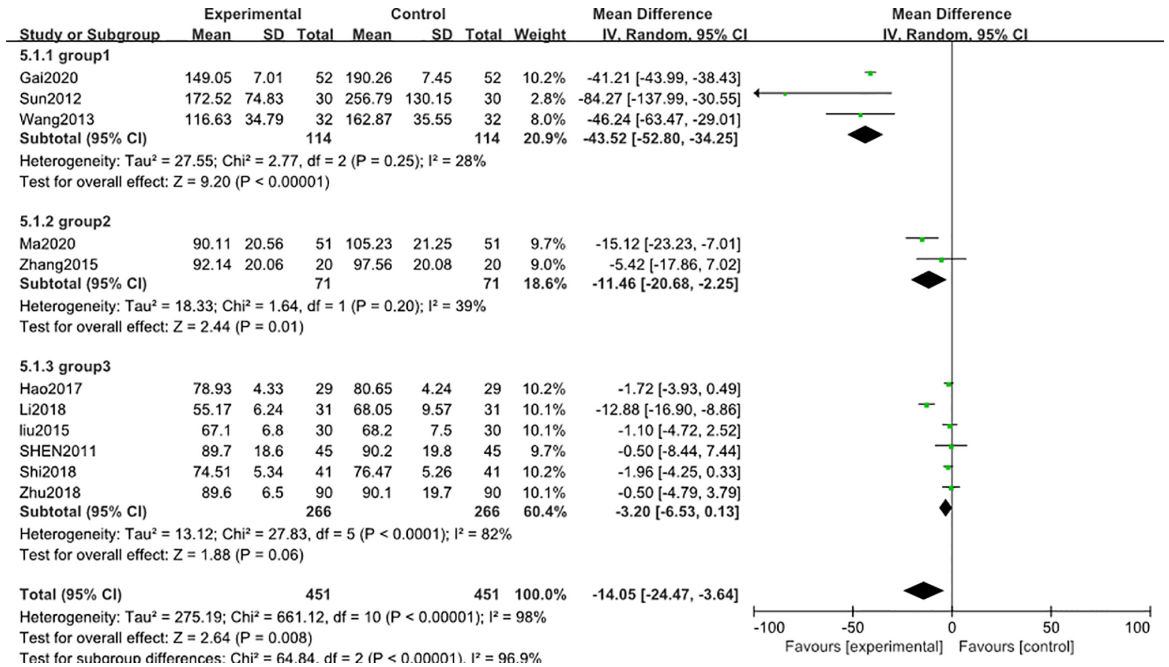

FIGURE 5 | Subgroup analysis of blood creatinine level after 3 months: comparison of treatment combined with TG against basic treatment (group 1: range 160-200 μmol/L, group 2: range 99-130 μmol/L, group 3: range 60-92 μmol/L).

Results With a Duration of 6-Month Treatment

Risk-of-Bias Assessment

The risk-of-bias assessment is shown in **Figure S5**. The adverse reaction outcome indicators were evaluated by the funnel plot, which showed no asymmetry (**Figure S6**).

24 h-UTP

7 studies (22, 48, 50, 52, 55, 56, 58) including 708 participants reported that the 24 h-UTP after 6-month of treatment combined with TG was significantly reduced [MD -0.91; 95% CI: -1.27 to -0.56; $I^2 = 92\%$] (**Figure 7**).

A subgroup analysis was conducted to reduce obvious heterogeneity. The studies were divided into 2 subgroups (group 1: range 2-3 g, group 2: range 4-5 g), according to the baseline level of 24 h-UTP. However, subgroup 1 still exhibited significant heterogeneity ($I^2 = 81\%$), while subgroup 2 involved only one article, thus could not be analyzed for heterogeneity. To explore the heterogeneity in subgroup 1, a *post hoc* sensitivity analysis was conducted, resulting in the exclusion of a trial reported by Kong et al. (48), thereby reducing the

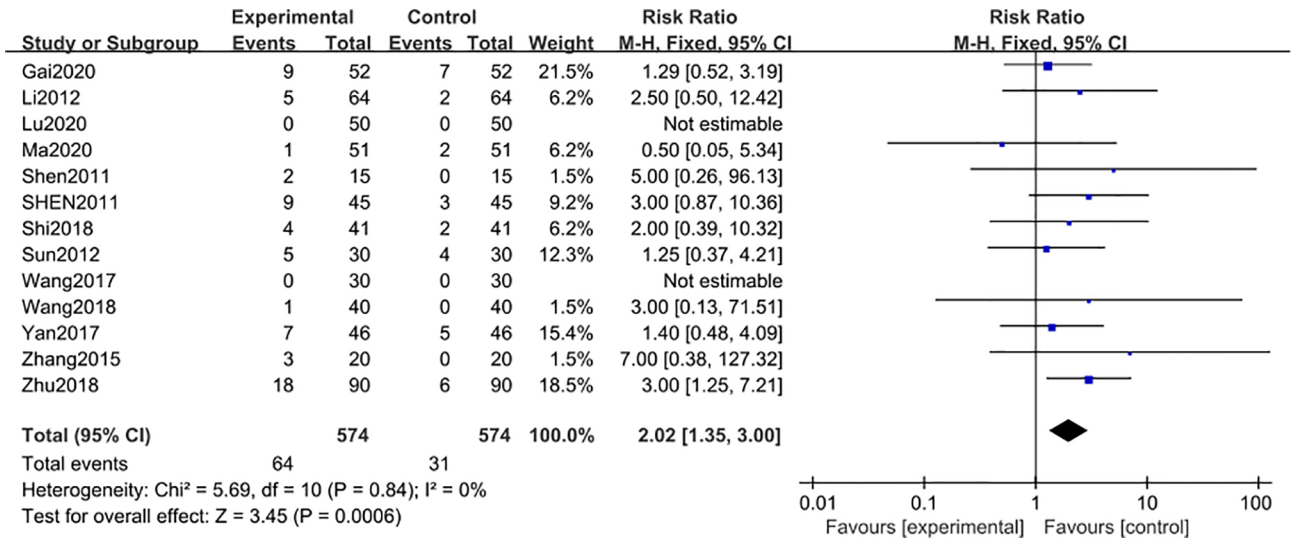

FIGURE 6 | Adverse reaction events after 3 months: comparison of treatment combined with TG against basic treatment.

TABLE 2 | Statistics of adverse reaction events after 3-month treatment.

adverse reactionevents	treatment combined with TGtotal (574 patients)	basic treatmenttotal (574 patients)
gastrointestinal reactions	29(5.1%)	25(4.4%)
leukopenia	17(3.0%)*	0(0.0%)
abnormal liver function	14(2.4%)*	0(0.0%)
hypotension	2(0.3%)	4(0.7%)
hyperkalemia	1(0.2%)	0(0.0%)
fatigue	1(0.2%)	0(0.0%)
elevated creatinine	0(0.0%)	1(0.2%)
dizziness	0(0.0%)	1(0.2%)
total	64(11.2%)	31(5.5%)

$p < 0.01$ (compared with basic treatment).

heterogeneity [MD -0.62; 95% CI: -0.77 to –0.47; I^2 = 42%] (**Figure 8**), suggesting that the difference in the 24 h-UTP baseline was the main source of heterogeneity, as was observed for 3-month treatment duration.

Blood Creatinine

8 studies (47, 48, 50, 52, 54–56, 58) including 780 participants reported that blood creatinine level was reduced after 6-month of treatment combined with TG [MD -2.85; 95% CI: -5.03 to -0.68%; I^2 = 87%], compared with the non-TG regular treatment (**Figure 9**). Similar subgroup analysis was used to reduce the heterogeneity to an acceptable degree after dividing the articles into two groups (group 1: range 70-88 μmol/L, group 2: range 94-109 μmol/L) (**Figure 10**), and the results showed that the heterogeneity was mainly sourced from variation at a baseline level of blood creatinine.

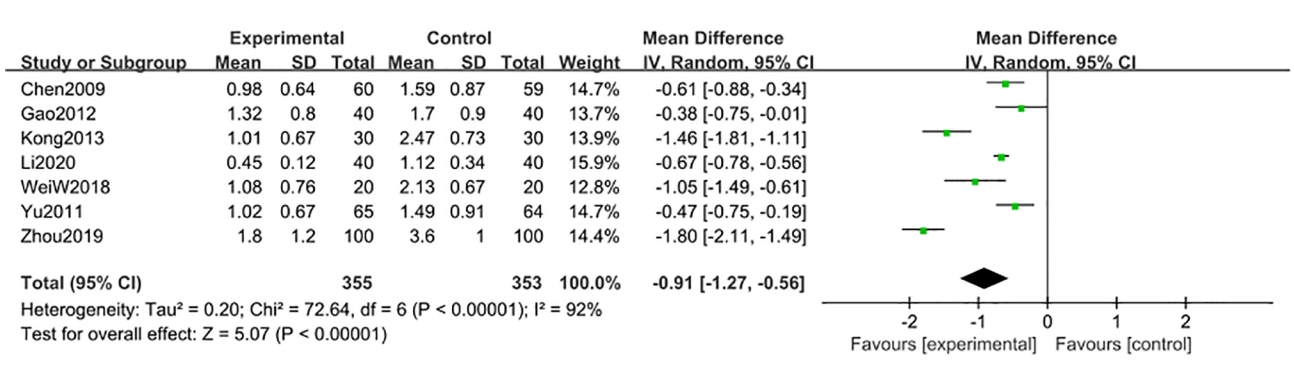

FIGURE 7 | 24 h-UTP level after 6 months: comparison of treatment combined with TG against basic treatment.

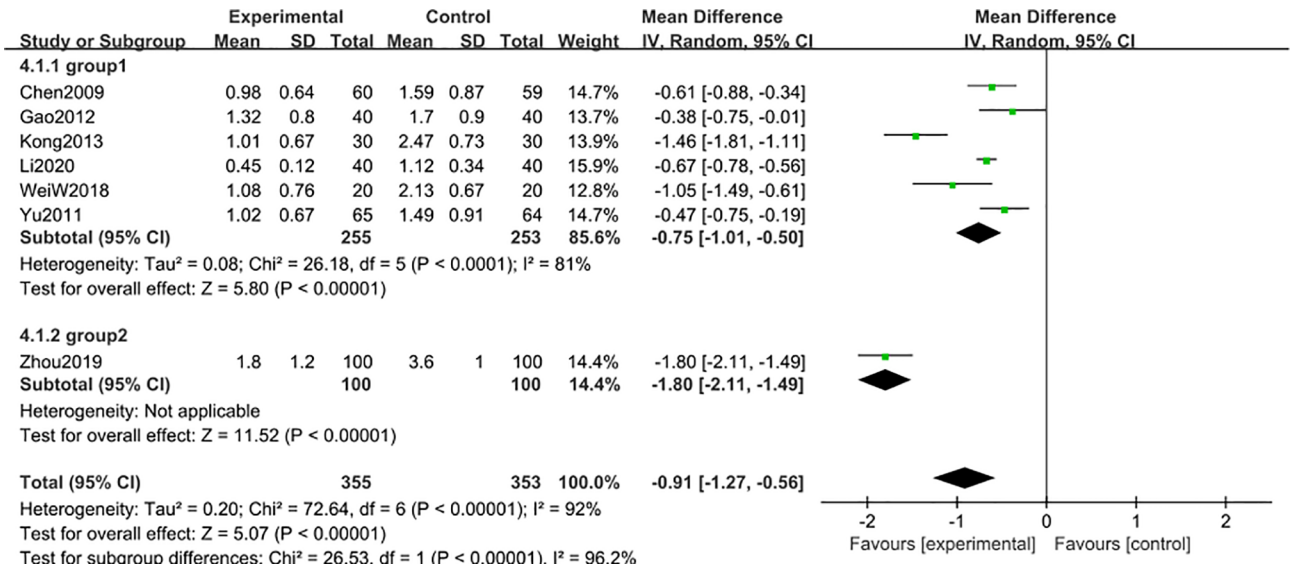

FIGURE 8 | Subgroup analysis of 24 h-UTP level after 6 months: comparison of treatment combined with TG against basic treatment (group 1: range 2-3 g, group 2: range 4-5 g).

Adverse Reactions

Eleven studies (22, 48–52, 54–58) including 1032 participants, reported the adverse reaction events after 6-month of treatment combined with TG. Compared with the regular treatment, TG treatment could significantly increase the adverse reaction events [MD 3.49; 95% CI: 1.96 to 6.22; $I^2 = 0\%$] (**Figure 11**). The results revealed that AEs after 6 months of TG treatment were mainly manifested as symptoms of leukopenia and abnormal liver functions, further confirming the results from the previous studies that long-term use of TG could lead to liver injury and leukopenia (**Table 3**).

GRADE Assessment

According to the Grading of Recommendations Assessment, Development, and Evaluation (GRADE), the quality of evidence for AE was moderate, while for 24 h-UTP and blood creatinine indicators were low (**Table 4**). Although the literature quality was not sufficiently high in this meta-analysis, however, a great number of participants were included in this study. This meta-analysis and systematic literature review, therefore, suggest that long-term application of TG could reduce the 24 h-UTP and blood creatinine level of patients with DN to normal levels, but at the same time, it can also induce considerable AEs, further complicating DN pathogenesis.

DISCUSSION

Supplement to Compendium of Materia Medica, an ancient traditional Chinese medical book authored by Zhao Xuemin

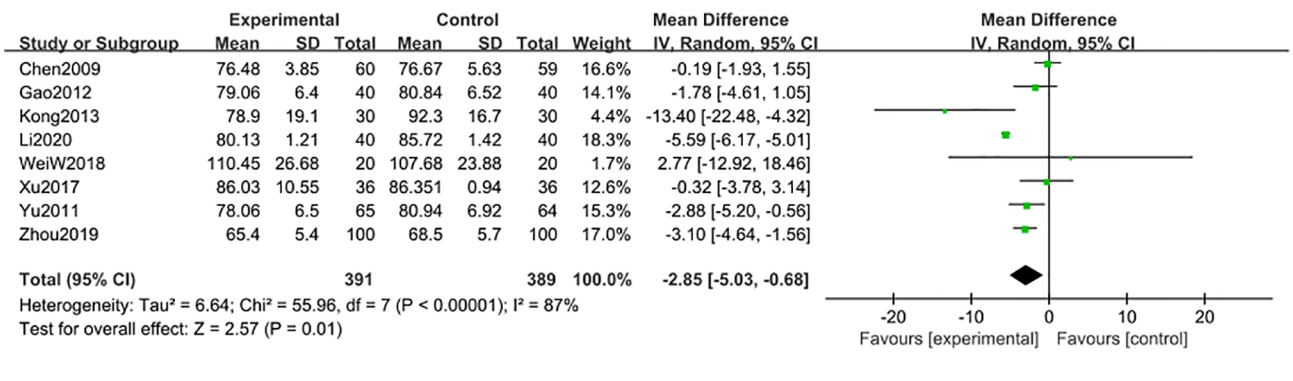

FIGURE 9 | Blood creatinine level after 6 months: comparison of treatment combined with TG against basic treatment.

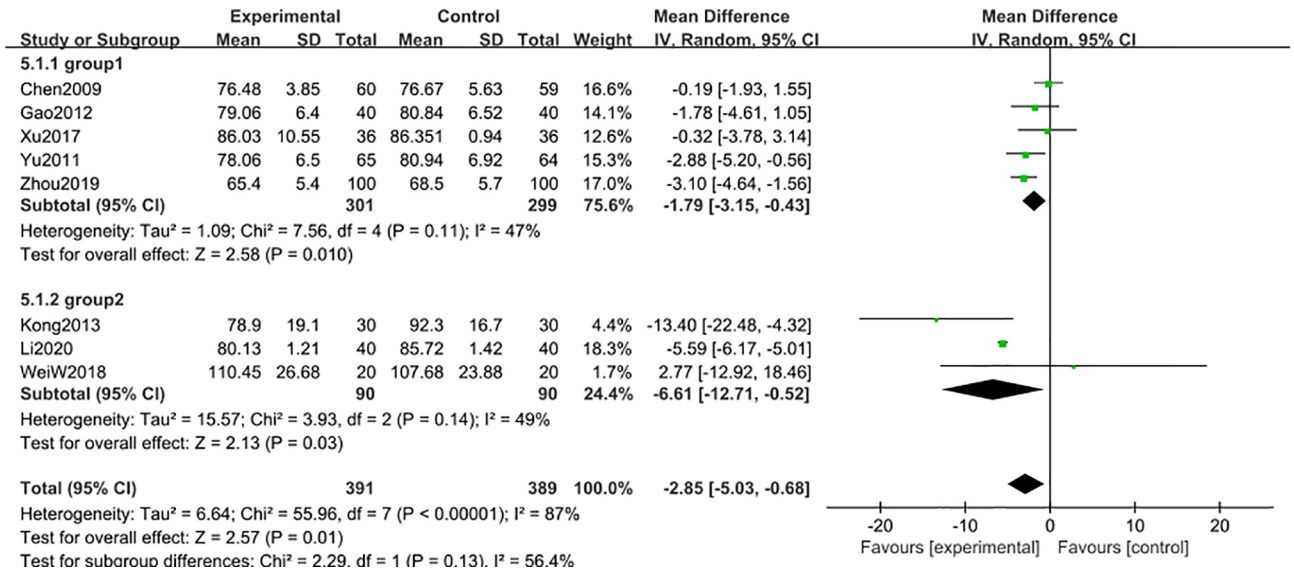

FIGURE 10 | Subgroup analysis of blood creatinine after 6 months: comparison of treatment combined with TG against basic treatment (group 1: range 70-88 μmol/L, group 2: range 94-109 μmol/L).

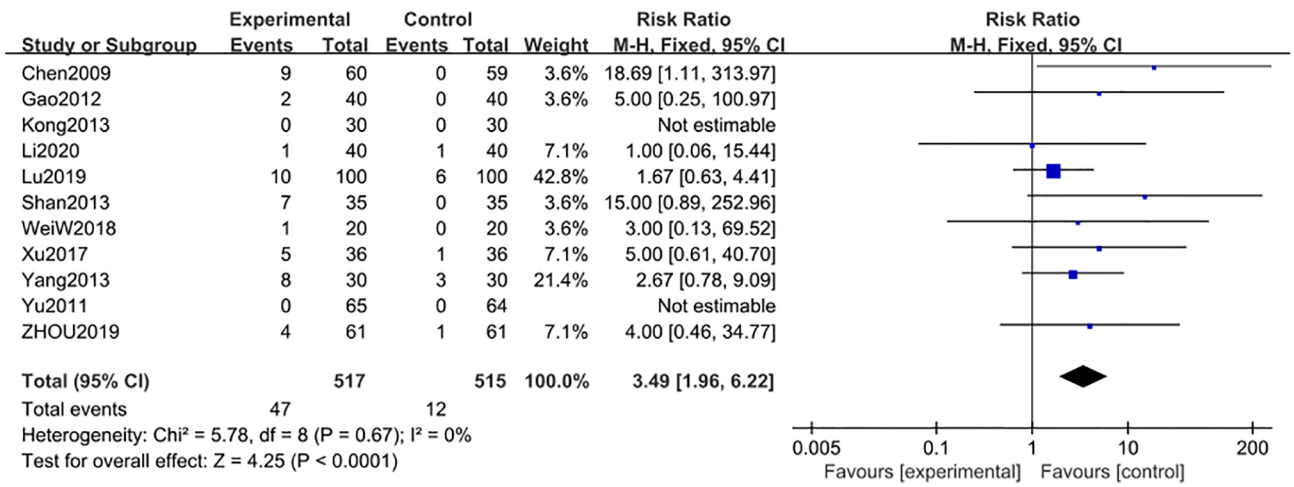

FIGURE 11 | Adverse reaction events after 6 months: comparison of treatment combined with TG against basic treatment.

(Qing dynasty) has documented that TW can be used in the treatment of tympanites, edema, epigastric fullness, jaundice, malaria that persists for a long time and also in traumatic injury (59), indicating that TW has been used as a critical traditional medicine in treating several fatal diseases for centuries. Notably, the possible toxicities of TW administration were also recorded at the beginning of its clinical applications (60).

In recent years, the active ingredient of TW extract, TG, has been purified and subsequently tested for its toxicity. Emerging studies have shown that TG could significantly lower the levels of 24 h-UTP and blood creatinine even in patients with the high initial baseline values, while the degree of reduction was smaller in patients with the lower initial baseline values, suggesting that TG may have a better efficacy for severe DN patients. Thus, though toxic, TG has still been used in clinical practice.

Studies on the long-term treatment effect show that after applying TG for DN 12 months, the 24 h-UTP of the patients were still significantly decreased (61). But the amount of relevant literature is insufficient to be included in this system reviews.

To maintain the balance between efficacy and safety profiles of TG usage, special attention should be paid to the appropriate dosage and duration of treatment course precisely depending on

TABLE 3 | Statistics of adverse reaction events after 6-month treatment.

adverse reactionevents	treatment combined with TGtotal (517 patients)	basic treatmenttotal (515 patients)
gastrointestinal reactions	4(0.8%)	2(0.4%)
leukopenia	14(2.7%)*	0(0.0%)
abnormal liver enzymes	25(4.8%)*	0(0.0%)
hypoglycemia	1(0.2%)	1(0.2%)
elevated creatinine	0(0.0%)	3(0.6%)
hyperkalemia	0(0.0%)	6(1.2%)
menstrual disorders	3(0.6%)	0(0.0%)
total	47(9.1%)	12(2.3%)

*$p < 0.01$ (compared with basic treatment).

the pathological symptoms of individual patients. Unfortunately, comprehensive and high-quality studies are still lacking for accurate clinical application of this important drug leading to controversial medicine guidance, clinical practice and random occurrences of adverse reactions. Although the medicine guidance recommends the course of treatment with TG should not be more than 3 continuous months, however, in several clinical investigations, patients have reportedly undertaken TG treatment for up to 6 continuous months.

Our results show that though AE profiles were very similar between 3-month and 6-month of course duration, the occurrences of severe AEs were relatively much higher after 6 months. Moreover, even after 3 months of TG treatment, severe AEs can happen at a rate as high as 5.4% of total patients, suggesting that better safety can be achieved by reducing the course duration even less than 3 continuous months. To our regret, there are not enough eligible research studies available on AE occurrence in relation to the duration of treatment with TG to allow us to comprehensively investigate the cause-effect relationship. Despite this, our work could still reveal that the published guidelines on the course of treatment with TG from both medicine guidance and clinical practice should be ameliorated, and more attention should be paid to the severe AEs related to TG medication, and symptomatic treatment should be applied immediately at the onset of these severe AE symptoms.

TW contains over 400 active ingredients. As an active ingredient of TW extract, TG's combination is simplified to a series of glycosides, which decrease the toxicity, but still lack of accurate pharmacological properties and manufactural quality. At present, the standard and proportion of TG in the market are lack of consistency, so the chemical composition produced by different manufacturers may be different (62). This may also contribute to the adverse reactions of TG.

In the absence of high-quality evidence for TG-associated adverse reactions, theories of traditional Chinese medicine practice stating that 'Stabilize that condition without excessive medical treatment' could be employed to adjust the appropriate dosage of TG, while the combination with herbal extracts containing leukocyte proliferation agents, e.g., *Cordyceps sinensis* (63), *Ganoderma lucidum* (64) and liver-protecting medicine, e.g., Milk thistle (65), *Polyporus umbellatus* (66) extracts may be an alternative therapeutic approach to alleviate AEs and improve the safety profile of TG application.

Taken together, this meta-analysis and systematic review suggest that more comprehensive and high-quality clinical investigations are urgently warranted to establish the treatment guidelines for TG and its related adverse reactions with respect to the patient's clinical stage of DN progression as well as comorbid symptoms to broaden the therapeutic application of this important natural medicine.

To further investigate the balance of efficacy and adverse effects, firstly, we would consider analyzing the differences among more course subgroups of DN patients and the relationships among clinical efficacy, AEs, and individual difference. Researchers should regulate the dosage, identify the manufacturers, and focus on the relationship between the duration treatment and AEs. Secondly, more clinical endpoints should be considered to evaluate the efficacy of TG, like Glomerular filtration rate (GFR).

From a real-world research perspective, investigators also need pay attention to the standard of randomization methods, blinding, and allocation concealment to standardize RCTs. At the same time, negative results should be properly reported to avoid publication bias. Further controlled studies should be done

TABLE 4 | GRADE assessment of quality of evidence for outcomes.

Duration (months)	Outcomes	Participants	studies	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	Overall quality of evidence
3	24h-UTP	1120	14	Serious[1]	No serious	NO serious	NO serious	Serious[2]	LOW[1,2], due to risk of bias, publication bias
3	blood creatinine	1002	12	Serious[1]	No serious	NO serious	NO serious	Serious[2]	LOW[1,2], due to risk of bias, publication bias
3	AR	1148	13	Serious[1]	No serious	NO serious	NO serious	NO serious	MODERATE[1], due to risk of bias
6	24h-UTP	708	7	Serious[1]	No serious	NO serious	NO serious	Serious[2]	LOW[1,2], due to risk of bias, publication bias
6	blood creatinine	780	8	Serious[1]	No serious	NO serious	NO serious	Serious[2]	LOW[1,2], due to risk of bias, publication bias
6	AR	1032	11	Serious[1]	No serious	NO serious	NO serious	NO serious	MODERATE[1], due to risk of bias

1.The random and blind methods were of poor quality. 2.There was publication bias.

on the age, stage, course of DN patients to evaluate what difference is made in the efficacy of TG for different populations.

LIMITATIONS

This study also suffers from certain inevitable limitations that require further consideration. Firstly, and most importantly, publications on the 24 h-UTP and blood creatinine indices after 3-month course of treatment with TG are significantly biased. And secondly, high-quality research studies relating to TG dosage and induced adverse reactions are not enough to firmly conclude on specific AEs due to particular TG treatment course.

Furthermore, most of the systematic reviews focused on efficacy or effectiveness. The methodology for conducting systematic reviews of beneficial effects from RCTs is well established, whereas the methods for systematically reviewing randomized or observational data on AEs are less well developed and less often used (67). Thus, researchers who conduct systematic reviews have limited sources of guidance, such as the suggestions offered by the Cochrane Collaboration. Moreover, the pre-determined harmful effects of interest were known to be under-reported in RCTs (68). These questions lead us to some innate limitations in this systematic review.

Although some researchers are accustomed to using 24 h-UTP and blood creatinine as the main surrogate biomarkers to evaluate the prognosis of renal disease in RCTs, 24 h-UTP and blood creatinine still have great limitations as clinical endpoint.

Because of the kidney's ability of compensate, when patients with renal impairment, the blood creatinine may still be in a normal level. Blood creatinine and 24 h-UTP does not reflect the long-time state of renal function well, so risks would be produced by using them to determine the efficacy. GFR is a better indicator for evaluating renal function. But, GFR is rarely used in clinical studies to evaluate the efficacy of TG. Recently, a growing number of studies have shown that the sensitivity of cystatin C to the decrease of GFR is better than that of blood creatinine, especially in the early stage of renal injury (69–71). Unfortunately, cystatin C is not widely applied at present, and relevant literature remain scarce. Our understanding of DN may greatly benefit from more detailed investigation into these surrogate indicators.

CONCLUSION

Our results have revealed that symptomatic treatments combined with TG can significantly lower 24 h-UTP and blood creatinine levels in DN patients than the basic treatment without TG can do, confirming the efficacy of TG. While forest plots of these two indicators have exhibited that apparent heterogeneity remains even after subgroup and sensitivity analyses, however, there are ways to reduce the heterogeneity to an acceptable degree.

Regarding the induction of adverse side-effects, patients from both 3-month and 6-month groups undertaking TG medications showed critical AE onsets, e.g. leukopenia and abnormal liver functions, especially more aggressively in patients of the 6-month treatment group. However, these results were inconsistent with the published reports we included in this study, indicating no significant differences in AE profiles between the experimental group and placebo-treated or control group. According to the GRADE assessments, the quality of evidence from these articles was low, primarily might be due to the insufficient sample size and error-prone experimental designs. Thus, the descriptions of AEs from the medicine guidance should be really concerning in clinical practice.

Importantly, the occurrence of AEs was very similar after 3-month (64/574, 11.1%) and 6-month (47/517, 9.1%) of TG treatment durations. However, the incidence of severe AEs after 6-month treatment with TG (39/517, 7.5%) reportedly had 39% increment than that happened after 3-month treatment with TG (31/574, 5.4%). The total percent of AEs in treatment with TG didn't greatly increase with TG, but as the course of treatment lasted, severe AEs were more likely to happen.

In summary, our work showed that TG was therapeutically effective in the treatment of DN-related symptoms like proteinuria, high serum creatinine, but insufficient sample sizes and inappropriate experimental designs caused non-significant AE differences between the experimental and control groups in several studies. AE occurrence rate was found nearly constant as the medicine duration increased, however, the percent of severe AEs after 6 months of treatment was 1.39 times more than that after 3 months.

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

YLi and RM wrote the manuscript. YLi and RM selected the trials. ZD and JZ extracted the data. YZ and LZ assessed the quality of the studies. ZH and YZ assessed the quality of the evidence. YLiu, PM, and YLi performed the statistical analysis. DH and YX conceived of the study, and all other authors critically reviewed the report. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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The Multi-Therapeutic Role of MSCs in Diabetic Nephropathy

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Diabetic nephropathy (DN) is one of the most common diabetes mellitus (DM) microvascular complications, which always ends with end-stage renal disease (ESRD). Up to now, as the treatment of DN in clinic is still complicated, ESRD has become the main cause of death in diabetic patients. Mesenchymal stem cells (MSCs), with multi-differentiation potential and paracrine function, have attracted considerable attention in cell therapy recently. Increasing studies concerning the mechanisms and therapeutic effect of MSCs in DN emerged. This review summarizes several mechanisms of MSCs, especially MSCs derived exosomes in DN therapy, including hyperglycemia regulation, anti-inflammatory, anti-fibrosis, pro-angiogenesis, and renal function protection. We also emphasize the limitation of MSCs application in the clinic and the enhanced therapeutic role of pre-treated MSCs in the DN therapy. This review provides balanced and impartial views for MSC therapy as a promising strategy in diabetic kidney disease amelioration.

Keywords: exosomes, therapy, diabetic nephropathy, hyperglycemia, mesenchymal stem cells

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INTRODUCTION

Diabetic nephropathy (DN) is one of the most common complications of Diabetic Mellitus (DM) (1). Parallel with the rising global prevalence of diabetes, DN often occurs after diabetic retinopathy, another microvascular complication of DM, presenting symptoms after 10 to 15 years of diabetes (2–4). The characteristics of DN are concluded as persistent proteinuria, reduced total glomerular filtration rate, raised arterial blood pressure, fluid retention, and shrunken kidney size (5–7). With intractable and refractory pathological progression, DN tends to progress into chronic kidney diseases (CKD). Almost half of people with type 2 diabetes will suffer CKD, as do approximately one-third of type 1 diabetes patients (8). Additionally, CKD always ends with end-stage renal disease (ESDR), leading to an extremely high rate of kidney transplantation and death (9–11). Up to now, the current medical treatment for DN still relies on pharmacological treatment aimed at glycaemic and blood pressure control, as well as kidney protection. Typical drugs like Chinese herbal medicine (12) and renin-angiotensin system-blocking medication (13) play a role while rarely change the outcome of DN. A study shows that 60.3% of patients being diagnosed with stage 4 CKD with DN rapidly progressed to ESRD or death (10.9%) after the treatment of angiotensin II type 1 receptor blocker (ARB) drugs and Rheum (13). Another data show over 200,000 deaths ascribed to advanced CKD/ESRD from 2003 to 2017 in the United States, and even with effective

drug treatment, 25% of people with type 2 diabetes and DN eventually develop ESRD (11). Despite this, poor prognosis of ESRD can be alleviated with early diagnosis and treatment of chronic kidney diseases (9). Thus, the poor prognosis of DN drives the efforts of many scientists to discover pathological mechanisms and effective therapy of DN.

Recently, increasing attention is being focused on mesenchymal stem cell (MSC) therapy. MSCs are specific types of cells under exploration for treatment of human diseases and have been found in tissues including adipose tissue (14), peripheral blood (15, 16), dental pulp (17), bone marrow (18), and neonatal tissues, especially in parts of the placenta (19) and umbilical cord (20, 21). The definition of MSCs involves three features: Self-renewal ability; Multi-differentiation potential; Specific surface biomarkers (22, 23). It had been shown that MSCs present with the capacity for self-renewal (24). Additionally, MSCs can differentiate into multiple cell types like chondroblasts (25), osteoblasts (26) adipocytes (27), and neuron-like cells (24) under specific induction. Over 95% of MSCs express surface markers CD73, CD90, CD105, while MSCs are negative for the expression of CD14, CD34, CD45, and human leukocyte antigen-DR (HLA-DR) (22, 28). Additionally, MSCs are capable of excreting small molecules, such as cytokines and exosomes. Owing to these unique features, MSCs appeal to researchers. Up to now, increasing numbers of studies concerning the therapeutic role of MSCs are ongoing. It had been reported that MSCs can alleviate disease progressions like stroking (29), myocardial infarction (30), and tumor (31). Furthermore, some clinic tests had made progress in the potential therapeutic role of MSCs.

MSCs derived exosomes, lipid membrane micro-vesicles with the size of 30-150nm, have been found to play a significant role in MSC therapy. Genetic molecules, including RNA (32, 33), and proteins (34, 35) can modulate micro-environments and epigenetic phenomena of organisms both in normal or pathological conditions. Thus, exosomes carrying numbers of these substances (36, 37), shuttling between cells and tissues, can transfer signals or materials and mediate micro-environmental communication in several types of diseases (38–40). Other studies have reported that cargo within MSCs derived exosomes mediates therapeutic approaches of diverse types of diseases, such as tumor (41), infections (42), metabolic diseases (43), and immune diseases (44).

Research related to MSC therapy is ongoing. Exploration concerning the therapeutic role of MSCs in DN, especially MSCs derived exosomes, are limited. This review covers the latest progress of MSCs treatment of DN, emphasizing the role of MSCs derived exosomes in these mechanisms and potential options for future therapies.

THE THERAPEUTIC MECHANISMS OF MSCS IN DN

DN often occurs during persistent high blood glucose in a DM patient, proceeding into CKD and ESRD. Hyperglycemia and kidney dysfunction are both therapeutic targets to alleviate the progress of DN. Accordingly, MSCs play a part in DN treatment mainly in two pathways, including hyperglycemia control and kidney impairment alleviation. MSCs can alleviate high blood glucose by promoting regeneration of islet cells and reducing insulin resistance, as well as improving islet function, thereby lessening the kidney injury resulting from high blood glucose. MSCs can also directly rescue kidney damage *via* diverse mechanisms. A bunch of studies demonstrated a phenomenon that MSCs treatment improved renal function by acting against inflammation, fibrosis, apoptosis as well as promoting angiogenesis. Only a portion of these mechanisms has been revealed, while the majority remains to be explored. The detailed aspects are shown in **Figure 1**.

The Role of MSCs in Blood Glucose Control

MSCs in the Regeneration of β Cells

MSCs present with potent potential for the regeneration of β cells. MSCs can differentiate into insulin-producing cells. Pan et al. found that the notch signal pathway of MSCs was highly inhibited under high glucose treatment *via* the methylation of notch-related genes, which suggested the directional differentiation of MSCs into functional β-cells (45). Another study also implied that insulin levels in circulation together with insulin-producing cells were increased after MSCs transplantation in diabetic mice model, suggesting MSCs are capable of differentiating into β-like cells (46). The types of MSCs that differentiate into insulin-producing cells are not limited, while the capability is not the same. Research indicated that although both BM-MSCs and subcutaneous adipose-derived MSCs can differentiate into islet-like clusters, BM-MSCs are superior to MSCs derived from adipose tissues in this process (47). Wharton's jelly-derived MSCs (WJ-MSCs), a type of perinatal stem cells with specific cell surface biomarker of EphA2, had also shown great potential in regeneration medicine (48). Previous research has focused on the transplantation of WJ-MSCs that had differentiated into islet-like cells *in vitro* (49, 50). However, a recent study revealed that even undifferentiated WJ-MSCs can migrate to the pancreas and differentiate into insulin-producing cells (51). Another research also reported a protocol to differentiate WJ-MSCs into pancreatic insulin-producing cells (52). At the same time, a clinical trial demonstrated that WJ-MSCs progressively decreased the glycated hemoglobin levels, fasting glucose level, and fasting serum C-peptide levels (53). A meta-analysis concerning six studies of WJ-MSCs therapy in 172 diabetic patients had demonstrated that WJ-MSC transplantation could improve HbA1c%, as well as C-peptide levels in both T1DM and T2DM (54). However, the number of included studies and the patients involved in most cases were quite limited, so further clinical studies are required to investigate the therapeutic efficacy of WJ-MSCs. Furthermore, MSCs promote endogenous β cell proliferation and replication. Apelin overexpression in MSCs leads to a significant expansion of β cell numbers and total pancreatic ß cell mass as well as enlarged islet size, implying the

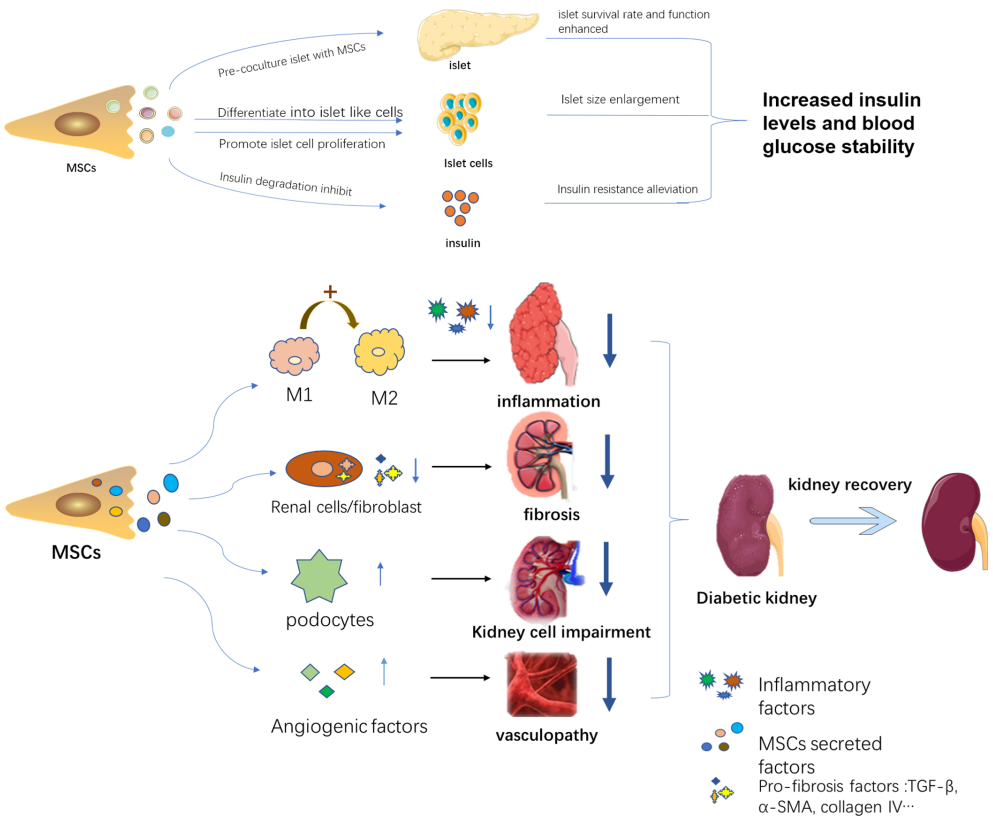

FIGURE 1 | The therapeutic mechanisms of MSCs in DN. MSCs alleviate DN progress in two pathways: 1. Decreasing blood glucose through islet function recovery, islet cell proliferation, and insulin sensitivity improvement. 2. Acting against kidney inflammation, fibrosis, and protect kidney-related cells and promote angiogenesis.

pro-proliferation effect of MSCs (55). Additionally, PI3K/Akt pathway inhibitors blocked the proliferation of β cells mediated by MSCs-conditioned medium, suggesting MSCs secretion induced β cell replication *via* the PI3K/Akt signal pathway (56). By these two ways, MSCs effectively promote islet β cell regeneration, thereby decreasing high blood glucose and its related hyperglycemia index.

MSCs in the Insulin Resistance

MSCs are also involved in the improvement of insulin sensitivity. Insulin resistance is another crucial point in the DM pathological process, especially in type 2 diabetes. Insulin resistance results in decreased insulin sensitivity, causing blood glucose to hardly back to a normal level and persistent hyperglycemia. Si et al. revealed that infusion of MSCs ameliorated hyperglycemia and proposed for the first time MSC therapy for improvement of insulin sensitivity (57). While the glucose-decreasing effect caused by a single infusion of MSCs was maintained only for a few days, further exploration found that multiple intravenous MSCs infusions reversed hyperglycemia and kept glycemia within normal levels (58, 59). As mentioned above, apelin may play vital roles in hyperglycemia remittance. Not only does it promote β cell proliferation, but apelin also increases insulin sensitivity. One study found that apelin-transduced WJ-MSCs rats shared faster glucose disposal and improved glucose tolerance compared to a placebo group (55). Several mechanisms had been reported to explain such improvement. Muscle mitsugumin 53 (MG53), a newly identified muscle-specific protein, is one pivotal element of insulin resistance in type 2 diabetes by participating in the insulin degradation process through insulin receptor substrate-1 (IRS-1) and the p-AKT pathway. MSCs infusion significantly inhibited MG53 elevation, subsequently restraining insulin-related factor degradation and alleviating insulin resistance (60). A clinical comparative study showed that DM patients with MSCs transplantation had an improved insulin sensitivity index, consequently resulting in a recession in demand of insulin doses. The declined area under curve (AUC) of 2^{nd}phase C-peptide response and restoration of IRS-1 expression in patients treated with MSCs provided further evidence for this therapy (61). Inflammatory cytokines and immune regulation also contribute to insulin resistance. Elevated inflammatory factors took part in insulin receptor destruction, exacerbating insulin resistance to a large extent. Sun X and colleagues observed raised NLRP3, L-1β, IL-18, and TNF-α expression in a type 2 diabetes mouse model and that this elevation could be blocked by MSCs

injection. The result implied MSCs could reduce inflammatory activities by downregulating the NLRP3-mediated inflammation pathway, accordingly alleviating insulin resistance (62). Macrophage polarization is another anti-inflammation approach for diabetes that enhances insulin sensitivity. Two distinct populations of macrophages have been discovered, including pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2). The research revealed that macrophages could be transformed from M1 to M2 in adipose tissue by the MSCs-activated IL-4R/STAT6/STAT3/PPARγ axis as well as MSCs-secreted monocyte chemoattractant protein-1 (MCP-1) and IL-6, improving inflammation and insulin sensitivity (63, 64). These results imply anti-inflammation is a crucial point in improving insulin sensitivity.

Immune regulation of MSCs also participates in blood glucose control *via* other mechanisms. MSCs provide a suitable environment for β cell survival through the regulation of some immune factors and cells. Boumaza et al. demonstrated that T cell cytokines were altered and the frequencies of $CD4^+/Foxp3^+$ and $CD8^+/Foxp3^+$T cells increased under MSCs treatment, enhancing β cell function (65).

As mentioned above, MSCs treatment could attenuate insulin resistance by decreasing inflammatory factors, regulating macrophage polarization and immune function.

MSCs in the Islet Dysfunction

There is additional evidence to show MSCs are competent to decrease blood glucose. In DM treatment, especially for type 1 diabetes, islet dysfunction is the key therapeutic focus. Traditional treatment around islet transplantation had been investigated for decades, but outcomes are unpredictable and ambiguous. Remarkably, the latest research revealed that MSCs can improve the function and survival rate of transplantation islets. Montanari et al. found that insulin secretion of the free islet was enhanced under MSCs treatment *via* the adhesion molecule N-cadherin, which improved survival and function of islets of Langerhans (66). WJ-MSCs, which contribute to the regeneration of β cells, were able to repair the destroyed islets as well by reducing the severity of insulitis in DM mice (51). Pre-culturing islets with a mixture of MSCs products put forward a perspective of cell-free therapy to improve clinical islet transplantation outcomes (67, 68). At the same time, researchers had demonstrated annexin A1 as playing an important role in this pathway (69). Another study also discovered enhanced glucose homeostasis under the co-transplantation of MSCs together with islets (70). Furthermore, such treatment effect of MSCs on islet can be improved under pre-hypoxic conditions (71). These results reveal that MSCs are beneficial for islet function improvement, suggesting MSC therapy as a prospect for hyperglycemia recovery.

The Role of MSCs in Kidney Impairment

The hyperglycemia control and islet cell protection in DN treatment work as effective ways to delay diabetes caused kidney impairment. However, direct protection and repairment toward kidney function are of more significance and efficiency. It has been shown that MSCs can regulate the immune environment, reducing fibrosis formation, and promoting angiogenesis. Additionally, the majority of these processes are accomplished by exosome-mediated paracrine function, which suggests that exosomes play a pivotal role in kidney function recovery (72).

The Role of MSCs in Anti-Inflammation and Anti-Fibrosis

The pathogenesis of DN is currently understood to be multifactorial, where inflammation appears to be relevant in the DN process, leading to metabolic disorder. Increasing research concerning inflammatory cell infiltration as well as pro-inflammatory cytokines secretion in DN pathogenesis gives a clue for DN treatment.

MSCs directly regulate immune cell migration and filtration, thereby reducing inflammatory activation. It is well-known that macrophages play an important role in the inflammatory process, and considerable research has focused on the macrophage. It had been demonstrated that MSCs-derived HGF inhibited MCP-1 expression to prevent macrophage infiltration (73). Lee et al. also found MSCs were associated with macrophage recruitment *via* expressing markers like C-C motif chemokine ligand 2 (Ccl2), vascular cell adhesion molecule-1 (VCAM1), and intercellular adhesion molecule-1 (ICAM1) (74). Similarly, research showed that the intravenous injection of MSCs reduced renal $CD68^+$ macrophage infiltration and inflammatory cytokine expression in the kidney of diabetic rats, and the fibrosis had been ameliorated (75). Meanwhile, the inductive effects of MSCs in macrophage polarization play a part in the impaired kidney as well. Lee's team realized increased expression of Arg1 in human umbilical cord blood MSCs could inhibit M1 polarization of macrophage, which decreased inflammatory factor secretion. Conditioned medium with human umbilical cord blood MSCs were able to rescue DN-induced mitochondrial mass reduction and mitochondrial reactive oxygen species (ROS) production compared to original adipose MSCs, which suggests that these effects were limited to umbilical cord blood-derived MSCs (74). Transcription factor EB (TFEB) expression was also found to be related to macrophage polarization. A study revealed that MSCs elicited macrophage transformation into the M2 phenotype *via* a TFEB-dependent mechanism. The transcription of TFEB activated the restoration of lysosomal and autophagy as well as mitochondrial bioenergetics of macrophages, which inhibited the pro-inflammation reaction (76). All these results suggested MSCs are capable of impacting macrophage function to inhibit inflammation activity.

The fibrosis and epithelial-mesenchymal transformation (EMT) had been regarded as a typical pathological change in DN as well, resulting in serious glomerular sclerosis and impaired filtration function. Research concerning the therapeutic role of MSCs in anti-fibrosis is ongoing and has achieved some promising results.

Interestingly, it seems like fibrosis and inflammation share several common pathways, as the treatment with MSCs tends to ameliorate fibrosis and inflammation together. Except for decreased inflammatory factors, collagen IV, α-SMA, and TGF-β in the kidneys of DN rats were decreased after MSCs treatment in the study of Xiang et al, which suggested MSCs can

inhibit fibrosis as well (77). Another study had demonstrated that Lipoxin A4 played a key role both in inflammation and fibrosis progression in DN pathogenesis. MSCs-derived Lipoxin A4 could reduce TGF-β as well as Smad2/Smad3 expression, a group of key factors attributed to extracellular matrix dysfunction, to rescue the fibrosis process. Meanwhile, three pro-inflammatory cytokines were decreased after MSCs-Lipoxin A4 injection, suggesting the pro-inflammatory actions had been inhibited by MSCs-derived Lipoxin A4 (78).

In conclusion, MSCs inhibit inflammatory reactions *via* impacting immune cell filtration. Additionally, EMT and fibrosis processes are delayed together with anti-inflammation of MSCs in DN.

The Role of MSCs in Podocytes Protection

Podocytes are regarded as the third layer of kidney filtration membrane structure, preventing protein loss from urine. Research has demonstrated that podocytes were decreased under persistent high glucose stimulus, which leads to albuminuria and proceeded to injure kidney function (79, 80). Thus, podocyte injury is an obvious pathological phenomenon in diabetes kidneys.

Several studies discovered that MSCs injection and transplantation could attenuate albuminuria and improve kidney function, which suggests that MSCs protect podocytes from dysfunction and injury. An animal study had demonstrated that rats treated with MSCs showed a suppressed increase in creatinine clearance rate and urinary albumin-to-creatinine ratio. Furthermore, the MSCs treatment reduced the loss of podocytes and podocyte markers and increased podocyte survival factor BMP-7 secretion (81). Since MSCs had been demonstrated to treat diabetic nephropathy, something must exist to help MSCs in this process, no matter from other mechanisms or MSCs themselves. Sun et al. revealed that stem cells from bone marrow relieved high glucose-induced podocyte apoptosis in combination with miR-124a *via* inhibiting the notch signal pathway (82). In a further study, they found that overexpressing miR-124a decreased the ROS production as well as cleaved caspase-3, bax, bcl-2, LC3-II/I, and p62 levels. These results suggested the activity of oxidative stress and autophagy of podocytes were significantly reduced by MSCs interfering together with miR-124a. Moreover, other researchers found secreted materials from MSCs also function in the treatment process. Li D and the team screened candidate factors in MSCs-conditioned medium and found that EGF levels were significantly increased, corresponding with lower podocyte apoptosis. At the same time, blocking of EGF decreased the therapeutic effects of MSCs-conditioned medium (83). This suggested that EGF together with MSCs could be regarded as a therapeutic target of DN progression.

To conclude, MSCs treatment can attenuate podocyte oxidative stress as well as podocyte death, thereby rescuing kidney dysfunction and slowing down the process of DN.

The Role of MSCs in Pro-Angiogenesis

Tissue reparation and neo-angiogenesis is another essential process in kidney renovation. Researchers found that medium conditioned with MSCs-secreted factors could induce angiogenesis.

Human embryonic MSCs have been found to rescue vascular damage in rats with CKD, and researchers thought that the conditioned medium of MSCs might make efforts in protecting vascular damage. The proteome profile of embryonic MSCs-conditioned medium showed that the presence of several gene products plays a role in angiogenesis and this effect had been subsequently identified in CKD rats. It had been shown that the average tube length was significantly increased in an angiogenesis assay after treatment with MSCs-conditioned medium, suggesting the MSCs-conditioned medium can promote vascular regeneration in the kidney (84, 85). However, this research failed to prove this effect was mediated by exosomes.

The Role of MSCs Derived Exosomes in DN

Exosomes, vesicles secreted by almost all types of cells, had been revealed to play a significant role in MSC therapy in DN. MSCs derived exosomes are involved in the alleviation of DN progress through aspects previously mentioned, including hyperglycemia control and kidney function protection.

The Role of MSCs Derived Exosomes in Blood Glucose Control

MSCs derived exosomes were found to alleviate insulin resistance and directly regulate glucose metabolism by induction of autophagy (86). Qin He and colleagues revealed MSCs derived exosomes participated in glucose homeostasis *via* autophagy-related AMPK pathway inhibition. In their research, the expression of glycolytic enzymes and lipolytic enzymes were increased after MSC-exosome treatment, whereas hepatic gluconeogenic enzymes were decreased; This suggests that MSCs derived exosomes were involved in the glucose metabolism to down-regulate hyperglycemia (87). Furthermore, MSCs derived exosomes increased the regulatory T-cell population and their products without a change in the proliferation index of lymphocytes in patients with moderate autoimmune type 1 diabetes, providing a suitable environment for β cell survival (88). Thus, MSCs derived exosomes ameliorate hyperglycemia *via* improved insulin sensitivity and β-cell function.

The Role of MSCs Derived Exosomes in Kidney Impairment

Exosomes derived from MSCs present a crucial role in kidney function repairment. Xiang et al. revealed that human umbilical cord-derived MSCs reduced inflammation both in DN rats and kidney cells. The mRNA expression of IL-6, IL-1β, and TNF-α was elevated in DN rats but was significantly decreased in MSCs-treated groups. To further identify these effects, Xiang et al. co-cultured MSCs derived exosomes with high-glucose-treated kidney cells, which included HK2 cells, NRK-52E cells, and hRGE cells; Results showed that MSCs derived exosomes suppressed high glucose-induced production of TGF-β, IL-6, IL-1β, and TNF-α in a dose-dependent manner. Moreover, several factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor

(HGF), and vascular endothelial growth factor (VEGF) were detected in MSCs derived exosomes, which suggests the anti-inflammatory effect was mediated by MSCs derived exosomes (77). Some studies found exocellular vesicles especially exosomes derived from MSCs had played a significant role in anti-fibrosis mechanisms. Some cohorts found DN mice treated with MSCs-derived extracellular vesicles presented improved kidney fibrosis, which suggested some specific patterns of miRNAs were involved in fibrosis (89). To be more specific, Ling Zhong's team revealed that MSCs-derived micro-vesicles shuttled miRNA-451a to down-regulate P15 and P19 expression, which assisted in restarting the cell cycle and slowed down the process of EMT, thereby regulating kidney fibrosis in DN (90). Other anti-fibrosis mechanisms had been revealed concerning the matrix-related proteins. MSCs treatment significantly decreased the proliferation of mesangial cells and upregulated matrix metalloproteinase (MMP) levels, which was related to extracellular matrix protein accumulation. MSC injection blocked myofibroblast trans-differentiation that resulted in reduced TGF-β1, fibronectin, and collagen I; These regulatory effects could be abolished by exosome consumption (91). This suggested that exosomes played a key role in ameliorating DN renal fibrosis. Additionally, autophagy had been shown to participate in the process of fibrosis development. One study found that MSCs derived exosomes reversed the diabetes-stimulated autophagy-related reduction in gene expression. Exosomes from MSCs could ameliorate the overexpression of TGF-β and fibronectin that were induced by autophagy inhibition, thus attenuating fibrosis, suggesting that these exosomes are capable of activating autophagy to protect renal function (92).

MSCs derived exosomes are involved in podocyte protection as well. Exosomes originating from adipose stem cells containing microRNAs powerfully impeded high glucose-induced migration and injury of podocytes. Remarkedly, several MSCs-derived exosomal microRNAs were found to participate in kidney cell protection. Adipose-derived stem cells secreted exosomes to adjust the survival of podocytes in the DN process. Mao et al. had discovered that microRNA-let-7a plays a protective role in renal cell apoptosis by targeting ubiquitin-specific protease 22 (USP22). Both elevated exosomal miR-let-7a or silenced USP22 reduced the apoptosis of renal cells and improved kidney function (93). Additionally, it had been demonstrated that the miR-251-5p inhibitor counteracted the improvement conferred by MSC exosomes on high glucose-induced proliferation inhibition and migration promotion of podocytes; And the miR-251-5p mimics significantly reversed the EMT process of the podocyte, suggesting exosomal miR-251-5p plays a role in podocyte protection (94). Meanwhile, miR-26a-5p took part in this process by targeting TLR4. Overexpression of miR-26a-5p inactivated the NF-κB pathway and downregulated vascular endothelial growth factor A (VEGFA) (95). Exosomal miR-16-5p from human urine-derived stem cells had been reported to alleviate DN *via* increasing podocyte viability and decreasing the rate of apoptosis. Overexpressed miR-16-5p in human urine stem cells significantly improved proteinuria as well as kidney function index (96). All this research concluded that miRNA could be adjusted to control the DN condition. MSCs derived exosomes are of great importance in the podocyte's protection, providing a novel perspective for DN therapy.

Another researcher investigated the pro-angiogenesis function of exosomes from MSCs. They repeatedly demonstrated the pro-angiogenesis function of MSCs-conditioned medium and identified that this potential was mediated by exosomes (97). Similarly, urine stem cell-derived exosomes contained increased VEGF, TGF-β, and angiogenin, which were reported to be involved in angiogenesis and cell survival (98). Up to now, most of these studies were limited to factor level detection, making the specific pro-angiogenesis mechanisms unknown. Notably, even though VEGF factor function had been verified to promote angiogenesis in other disease models, the function of VEGF is still undefined in DN since it had been reported to increase glomerulus permeability and proteinuria (99–101). There are few studies focused on the mechanism of VEGF derived from MSCs derived exosomes in DN models, which make the function of VEGF still puzzling in the DN process.

Overall, the specific functions of MSCs from different origins in kidney protection are covered in **Table 1**. The factors released by MSCs as well as involved DN models in kidney function recovery are listed.

LIMITATION AND POTENTIAL OF MSCS THERAPY IN DN

Limitation of MSCs Therapy

MSCs present an excellent therapeutic effect on renal function alleviation, which offers the desired perspective for novel DN therapy. However, the progression of passing MSCs therapy from the bench to the bedside has been very slow for several reasons. The quantity and quality of MSCs are the most challenging for clinic application. For the quantity, even though the procedure for MSCs isolation and expansion into a nonclonal population of stromal cells had been standardized according to the International Society for Cell & Gene Therapy (ISCT), MSCs originate from different donors or even different tissues have diverse proliferation rates and capability. Meanwhile, every nonclonal population of MSCs may contain a different proportion of stem cells, which may affect the biological properties of the total population. Therefore, the percentage of stem and progenitor cells in each batch of MSCs must be evaluated exactly before being used in patients (105). For the quality, MSCs ex vivo expansion results in cell senescence inevitably, which will decrease the capability of MSCs, including differentiation ability, migration ability as well as regeneration ability (106, 107). Another issue that must be considered is the safety of MSCs transplantation. Although some studies had proved the efficacy of MSCs in DM, which had been listed in **Table 2**, the numbers of clinic studies and involved patients of MSCs therapy were limited, thus the efficacy was unsure and

TABLE 1 | The detailed function of MSCs secreted factors in diabetic nephropathy.

MSCs Original	Model	Secreted Factors	Function	Reference
Human umbilical cord	DN rats; HK2 cells, NRK-52E cells, hRGE cells	EGF, FGF, HGF, VEGF	Anti-inflammation and fibrosis	(77)
Human umbilical cord	Rhesus macaque; HK2 cells	IL-16	Anti-inflammation and fibrosis	(102)
Bone Marrow	DN rats;	HGF	The expression of MCP-1 could be inhibited *via* MSCs secreted HGF, thereby reducing macrophages infiltration, and pro-inflammatory cytokines	(73)
Human umbilical cord	Mice; RAW264.7 cells	Arg1	Arg1 suppress M1 polarization and improve macrophage mitochondrial function, thereby inhibiting inflammation	(74)
Bone Marrow	DN rats; Peritoneal macrophages	–	suppressed renal macrophage infiltration and inflammatory cytokine secretion	(75)
Bone Marrow	DN mice; Peritoneal Mφ	TFEB	TFEB mediate macrophage transfer into M2 to promote anti-inflammatory reaction	(76)
Bone Marrow	DN rats; Glomerular mesangial cell	Lipoxin A4	Lipoxin A4 suppress fibrosis viatargeting TGF-β/smad axis; Anti-inflammation	(78)
Human umbilical cord	DN mice; HK2 cells	miR-451a	Down the expression of α-SMA, P15INK4b, and P19INK4d to inhibit EMT process and restart cell cycle, thereby slowing fibrosis.	(90)
Mouse umbilical Cord	DN mice; Mouse mesangial cell	—	Exosomes from MSCs reduced the fibronectin and collagen expression *via* inhibiting myofibroblast trans-differentiation triggered by TGF-β1 and cell proliferation mediated by PI3K/Akt and MAPK signaling pathways and elevating the levels of MMP2 and MMP9.	(91)
Bone Marrow	DN rats	—	MSCs-exosomes increased autophagy markers mechanistic target of rapamycin (mTOR), Beclin-1 as well as light chain-3 (LC-3) to activate autophagy, thus improve renal fibrosis.	(92)
Bone Marrow	DN rats; Renal cell	miR-let-7a	Increased miR-let-7a in MSCs-exosomes reduced blood urea nitrogen (BUN) and serum creatinine (SCr), blood lipid-related indicators total cholesterol (TC) and triglyceride (TG), renal cell apoptosis by repressing USP22 expression	(93)
Bone Marrow	DN Rats;	—	MSCs injection promoted podocytes to express higher levels of BMP-7, and improved kidney function	(81)
Bone Marrow	DN rats; Murine podocytes	miRNA-124a	MSCs combined with miRNA-124a down-regulate the expression of Notch1, NICD, Hes1 and Delta to reduce podocytes apoptosis.	(82)
Bone Marrow	podocytes	miRNA-124a	Overexpression of miRNA-124a decreased the intensity of oxidative stress and autophagy of podocytes *via* the PI3K/Akt/mTOR pathway	(103)
Umbilical Cord	DN rats;	—	MSCs up-regulated anti-apoptosis proteins expression and suppressed apoptosis signal regulating kinase 1 and P38 MAPK	(104)
Adipose	DN mice podocyte	EGF	EGF increased in MSCs condition medium to attenuate podocyte apoptosis.	(83)
Adipose	DN mice MPC5 cells	miR-215-5p	Exosomes from adipose stem cells containing mir-215-5p to inhibit EMT of podocytes *via* zinc finger E-box-binding homeobox 2 (ZEB2)	(94)
Adipose	DN mice MP5 cells	miR-26a-5p	Adipose MSCs-exosomes containing mir-26a-5p attenuate kidneys cells injury *via* targeting Toll-like receptor 4 (TLR4).	(95)
urine	DN rats podocytes	miR-16-5p	Overexpression of miR-16-5p in urine stem cells exosomes inhibited VEGFA expression to confer protective effects on human podocytes	(96)
Urine	DN rats podocytes	VEGF, TGF-β1, angiogenin	The VEGF, TGF-β1, and angiogenin might be related to angiogenesis.	(98)

hardly applied in the clinic. Additionally, some clinic experiments of MSCs in other diseases demonstrated that MSCs will boost cancer growth. A study indicated the expression of VEGF in tumor cells as well as the activation of RhoA-GTPase and ERK1/2, were increased after human MSCs condition medium treatment (118). Another research reported that gastric cancer MSCs promoted immune escape by secreting IL-8, inducing programmed cell death ligand 1 (PD-L1) expression in gastric cancer cells (119). It seems that MSCs contribute to tumor cell growth and tumor development. The relationship between MSCs and tumor cells is still unknown, leaving a great challenge for MSCs therapy application.

The Potential of MSCs Therapy in DN

MSCs from diverse donors with different capability as mentioned, the ability of MSCs from healthy people are superior to that from patients. While autologous MSCs with less immunological rejection shows better potential than MSCs from other individuals, which contradicted with the impaired regeneration and function of autologous MSCs (120, 121). Therefore, some research has focused on MSCs modification and co-culture to increase the MSC capacity in cell therapy. Pre-treatment of MSCs with specific substances as well as the growth environment had been revealed to enhance the MSCs therapeutic effect in DN. The general pathways had been shown in **Figure 2**.

TABLE 2 | The clinic trials of MSCs therapy in DM.

MSCs origins	Number of patients	The key findings	Follow-up period (year)	years	references
BM-MSCs	30 (BM-MSCs: 10 BM-MNCs: 10 Control: 10)	Both BM-MSCs and BM-MNCs therapies in T2DM result in significant decreases in insulin dose requirement accompanied by improvement in insulin sensitivity and β-cells function	1	2017	(61)
Umbilical cord-MSCs	42 (UC-MSCs/ BM-MNCs: 21Control: 21)	MSCs/MSCs treatment cause progressive reductions in insulin dose requirements and HbA1c levels and increased fasting C-peptide levels as well as $AUC_{C\text{-}Pep}$	1	2016	(108)
WJ-MSCs	61 (WJ-MSCs:31 Control:30)	Blood glucose, glycosylated hemoglobin, C-peptide, homeostasis model assessment of pancreatic islet β–cell function, and incidence of diabetic complications in the MSCs group were significantly improved when compared with the control group during the 36 months follow–up in T2DM	3	2016	(109)
WJ-MSCs	12 (liraglutide +WJ-MSCs:6 liraglutide:6)	liraglutide treatment in combination with WJ-MSCs improves glucose metabolism and the β cell function in T2D patients	6 months	2016	(110)
Adipose-MSCs	20 (AD-MSCs:10 Control:10)	Variable and sustained improvement in mean fasting blood glucose(FBG), post-meal blood glucose(PBG), HbA1c, and serum C-peptide was noted after the treatment of insulin-secreting mesenchymal stromal cell.	2	2015	(111)
BM-MSCs	20 (MSCs:10 Insulin treatment:10)	Autologous MSC treatment of new-onset type 1 diabetes may be a safe and feasible strategy to intervene in the disease process and preserve β-cell function	1	2015	(112)
WJ-MSCs	6	Following transplantation, no immediate or delayed toxicity associated with the cell administration, and the levels of fasting C-peptide, the peak value and the area under the C-peptide release curve increased significantly within one month and remained high during the follow-up period	2	2015	(113)
Umbilical cord-MSCs	18	FBG and PBG were significantly reduced and plasma C-peptide levels and regulatory T (Treg) cell number were numerically higher after UMSC transfusion in T2D patients.	6 months	2014	(114)
WJ-MSCs	22	WJ-MSC transplantation decreased the level of HbA1c, increased the level of fasting C-peptide, decreased the FBG, 2h-postprandial blood glucose level, insulin requirement, and oral hypoglycemic drugs; and reduced the systemic inflammation and T lymphocyte counts in patients with T2DM	1	2014	(53)
WJ-MSCs	29 (WJ-MSCs:15 Control:14)	No reported acute or chronic side effects in the MSCs group compared with the control group, both the HbA1c and C peptide in MSCs group patients were significantly better than either pre-therapy values or control group patients during the follow-up period in T1DM.	2	2013	(115)
Placenta-MSCs	10	The mean levels of insulin and C-peptide at each time point in a total of 10 patients were higher and the renal function and cardiac function were improved after MSCs infusion, indicating that transplantation of placenta-MSC represents a simple, safe and effective therapeutic approach for T2D patients with islet cell dysfunction	1	2011	(116)
Adipose-MSCs	11	Transplantation of insulin-secreting cells that differentiated from AM-MSCs decreased insulin requirement and Hb1Ac levels and serum C-peptide levels were improved in T1D patients.	2	2010	(117)

The angiotensin-converting enzyme 2 (ACE2) plays a protective role in DN patients *via* degrading Ang II into Ang2-7, thus alleviating the detrimental effects of Ang II. Liu Q et al. found that ACE2-modified MSCs showed superior amelioration on glomerular fibrosis in DN compared to MSCs alone. After co-culturing ACE2 with MSCs, the expression of ACE2 was obviously higher and MSCs-ACE2 treatment groups showed reduced levels of collagen I as well as TGF-β mRNA and protein. The pre-treatment had diverse effects on the expression of angiotensin receptor (ATR). The injection of MSCs-ACE2 did no effect on the expression of AT1R, while the expression of AT2R increased; This increase in the MSCs-ACE2 group was greater than that in either the MSCs group or the ACE2 alone, which gives speculation that elevated AT2R is involved in the renal protective effect of MSCs-ACE2 treatment (122).

Melatonin (MT) is a neurohormone mainly secreted by the pineal and non-pineal cells and has demonstrated powerful antioxidative and anti-inflammation properties for kidney diseases like acute kidney injury (AKI) as well as CDK. MSCs treated with MT also had a significant effect on DN treatment. Rashed et al. discovered that MSCs treated with MT showed positive effects in a DN model. Respectively, the increased TNF-α, and decreased TGF-ß, IL-10, and SOD corresponded with improved antioxidative, anti-fibrosis, and anti-inflammation effects. Additionally, MT pre-incubation significantly increased the cell proliferation of MSCs *in vitro* (123). Other research found that cellular prion protein (PrP^C) mediated the functional recovery of MSCs. A team observed that MT-treated CKD-MSCs had a longer survival rate and alleviation of senescence. Furthermore, they found PrP^C was overexpressed after MT treatment. Enhanced mitochondrial activity, as well as MSCs functional recovery, corresponded with MT treatment. PrP^C knockdown significantly neutralized the benefits from MT-MSCs treatment, suggesting the alleviation effects were mediated by PrP^C (124). Focusing on the MSCs functional rescue research, it was shown that MSCs treated with MT-derived exosomes had been discovered to transfer microRNAs to stimulate the increase of PrP^C, thereby recovering MSCs functions (125). The team developed and finished a complete logical story of how MT affects the function of

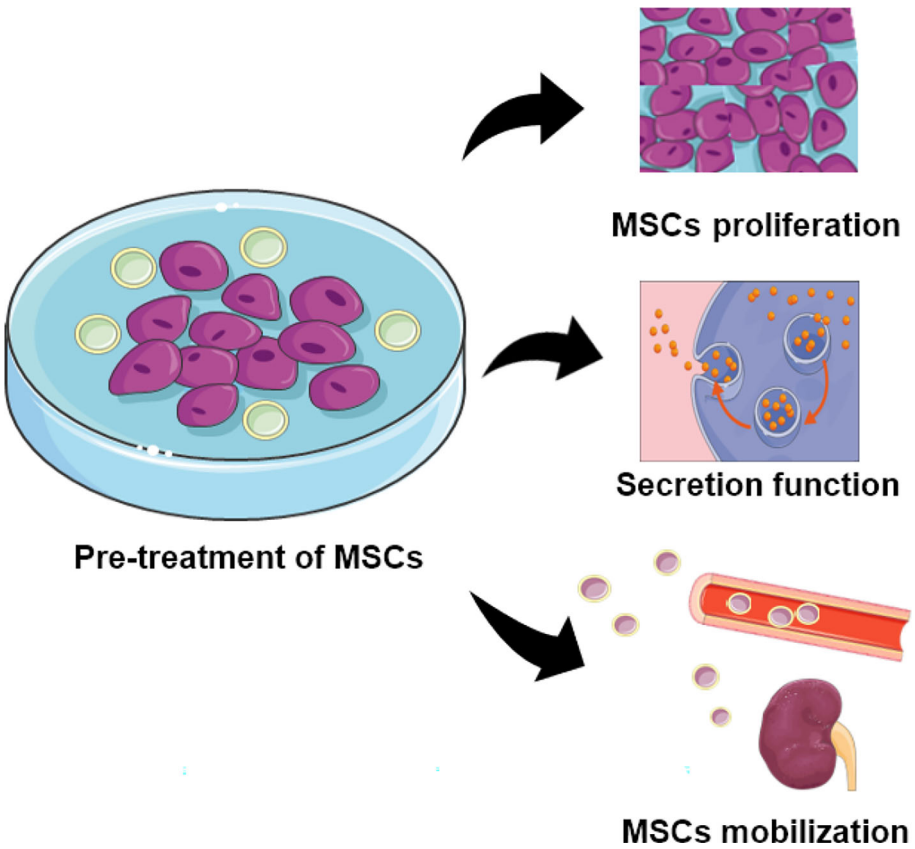

FIGURE 2 | The function of MSCs pretreated with specific substances. Pre-treated MSCs demonstrate increased capability for proliferation, secretion, and localization.

MSCs. MT possesses the ability to enhance MSCs capabilities and demonstrates the potential for constructive effects with MSCs-based therapy in DN. Additionally, factors including clinical drugs and other biological hormones were involved in PrP^C expression (126–128), providing a promising approach for PrP^C expression to enhance MSCs function.

Umbilical cord extract, namely Wharton's jelly extract supernatant (WJs), which contains several types of biologically active substances including growth factors, cytokines, extracellular matrixes, and exosomes, provides a suitable survival environment to maintain MSCs properties. By culturing with WJ, the morphology, proliferative ability, and cell mobilization of BM-MSCs in a DN model increased to a large extent. Meanwhile, the mitochondrial degeneration and abnormal expansion of the endoplasmic reticulum (ER) were improved as well. As for the mechanism, exosomes secreted by WJ might be the key factor to activate DM-MSCs, since WJ-derived exosomes showed similar effects on MSC function compared with WJ (129).

CONCLUSION

Increased prevalence and low therapeutic effects of diabetes make kidney impairment inevitable. Similarly, ineffective treatment of DN often ends with CKD and ESRD, which lead to kidney transplantation and even death. The MSCs-based cell therapy brings a prospective treatment for DM as well as DN. It had been reported that MSCs were involved in blood glucose reduction, anti-inflammation, anti-fibrosis, podocyte protection, and pro-angiogenesis processes in DN. Furthermore, researchers investigated the mechanisms of MSC therapy and found that exosomes play a significant role in MSC therapeutic effects. Exosomes serve as a vehicle, transmitting a variety of substances from MSCs to recipient cells, especially microRNAs; This may confer positive effects to recipient cells. Hopefully, autologous MSCs with little immunological rejection is of more significance than MSCs from other origins in DN treatment. However, kidney injury is regularly accompanied by impairment of MSCs function, resulting in lower therapeutic effectiveness of autologous MSCs. Studies concerning MSCs functional recovery emerged under this situation. Factors including clinical drugs and hormones have been involved in the MSCs functional recovery *via* improving MSCs growth and secretory capabilities.

Even with some challenges for MSCs therapy in clinic application, MSCs-based cell therapy offers a bright future for DN treatment. Exosomes from MSCs as well as pre-treatment of MSCs can be regarded as a key breakthrough for improving

therapeutic efficiency. More clinical trials are required to identify the efficacy of MSCs in DN.

AUTHOR CONTRIBUTIONS

L-QY: manuscript writing and approving final version of manuscript. YW: study conduct, data analysis, and manuscript writing. S-KS, BG, FL, M-HZ, L-ML, Q-SX and UM: data analysis. All authors contributed to the article and approved the submitted version.

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REVIEW
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Current Challenges and Future Perspectives of Renal Tubular Dysfunction in Diabetic Kidney Disease

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Over decades, substantial progress has been achieved in understanding the pathogenesis of proteinuria in diabetic kidney disease (DKD), biomarkers for DKD screening, diagnosis, and prognosis, as well as novel hypoglycemia agents in clinical trials, thereby rendering more attention focused on the role of renal tubules in DKD. Previous studies have demonstrated that morphological and functional changes in renal tubules are highly involved in the occurrence and development of DKD. Novel tubular biomarkers have shown some clinical importance. However, there are many challenges to transition into personalized diagnosis and guidance for individual therapy in clinical practice. Large-scale clinical trials suggested the clinical relevance of increased proximal reabsorption and hyperfiltration by sodium-glucose cotransporter-2 (SGLT2) to improve renal outcomes in patients with diabetes, further promoting the emergence of renal tubulocentric research. Therefore, this review summarized the recent progress in the pathophysiology associated with involved mechanisms of renal tubules, potential tubular biomarkers with clinical application, and renal tubular factors in DKD management. The mechanism of kidney protection and impressive results from clinical trials of SGLT2 inhibitors were summarized and discussed, offering a comprehensive update on therapeutic strategies targeting renal tubules.

Keywords: renal tubular dysfunction, tubular biomarkers, sodium-glucose cotransporter-2, diabetic kidney disease, therapeutic strategies

INTRODUCTION

Along with the disease spectrum that evolved around the world over the past 30 years, diabetic kidney disease (DKD) has become the leading cause of end-stage kidney disease (ESKD) at daunting rates in both developed and developing countries (1, 2). Due to the increased risk of morbidity and mortality of DKD, the number of DKD related studies rapidly increased over the past two decades,

with more than 27,500 papers published from 2000 to 2017 (3). Growing evidence suggests the underlying pathogenesis of DKD involves the renal proximal tubular epithelial cell dysfunction in a high glucose environment, oxidative stress, inflammation, fibrosis, and apoptosis (4). In addition, a large number of tubular biomarkers for DKD screening, diagnosis, and prognosis are tightly associated with the prognosis of the kidney in DKD, providing evidence for potential shifting of the paradigm from glomerulocentric to tubulocentric theory (5). It has been repeatedly shown that compared with the glomerular lesions, the extent of tubulointerstitial lesions correlates well with renal function, and the associated biomarkers have been identified (6). Urinary tubular injury markers may increase in patients with diabetes even before the onset of microalbuminuria (7, 8). Plasma tubular markers, which may reflect inflammatory and fibrotic responses, oxidative stress, and capacity of reabsorption in DKD, were also reported to be associated with early renal function decline and DKD progression (9, 10). Moreover, the biomarkers of tubulointerstitial function and structural changes were ultimately proven to be better predictors of disease progression and long-term prognosis than the current markers (11). Current prognostic markers of DKD have certain limitations. Estimated glomerular filtration rate (eGFR) and albuminuria are only modestly useful for risk prediction in type 2 diabetes mellitus (T2DM) patients with preserved renal function, and DKD progresses even in the absence of albuminuria (12, 13). Most importantly, inhibition of proximal tubule glucose transport *via* sodium-glucose cotransporter-2 (SGLT-2) has shown nephroprotective effects in a variety of large-sample, multicenter, placebo-controlled, and randomized clinical trials. By investigating the mechanism of the newest disease-modifying treatments for DKD, an accumulating body of research had documented the vital role of tubule function in regulating glomerular filtration through tubuloglomerular feedback. Moreover, the growth of the proximal tubule in the diabetic context supplies muscular strength to the established status of renal tubules in DKD (14, 15). The tubuloglomerular feedback mechanism begins with the theory that diabetic hyperfiltration and glomerular capillary hypertension are significant treatable stressors contributing to the progression of DKD (16–19). In diabetic conditions, the increased filtered load of glucose results in an increase in sodium-coupled glucose reabsorption by the proximal tubule. Decreased sodium delivery to the macula densa subsequently inhibits adenosine-triphosphate (ATP) conversion into adenosine, which results in the vasodilation of the afferent arteriole and the intrarenal activation of the renin–angiotensin–aldosterone system (RAAS), ultimately inducing glomerular hypertension and kidney hyperfiltration (15, 16). Hence, hyperreabsorption of water and solutes has a central role in the regulation of eGFR, highlighting the importance of alteration in the tubuloglomerular feedback for the development of DKD.

This review aimed to summarize the latest updates on the pathogenesis of renal tubular dysfunction in DKD, potential applications of tubular biomarkers, and renal tubule-targeting therapeutics based on evidence from recent trials in DKD.

NEW INSIGHTS INTO THE PATHOPHYSIOLOGY OF RENAL TUBULES IN DKD

Morphological Changes

Recently, there has been a growing consensus that tubular abnormalities, a consistent feature of DKD, are not the aftermath of glomerular damage. Tubular cells have the potential to be the primary targets for diverse pathophysiological influences (20). The shift has been suggested from the traditional paradigm of glomerulus-centered pathophysiology extended to the tubule-interstitium (21, 22). Morphological changes of tubulointerstitial lesions in DKD include thickening of the tubular basement membrane, tubular atrophy, interstitial inflammation, interstitial fibrosis, and vascular abnormalities (23). The association of tubulointerstitial lesions with DKD progression has been validated in several reports. A study in a Chinese population with an early stage of biopsy-proven DKD suggested that interstitial lesions and glomerular injuries were independently predictive of the time to ESKD (24). Another study from the United States population at relatively late stages of biopsy-proven DKD showed that interstitial fibrosis and tubular atrophy were of univariate significance for their ability to predict clinical prognosis (25). Moreover, the association of histological lesions with renal progression may differ in type 1 and type 2 DKD. In type 1 diabetes mellitus (T1DM), glomerular damage was observed through all stages. Nevertheless, minimal or no glomerular lesions but notable tubulointerstitial and/or arteriolar abnormalities were observed in type 2 DKD patients with microalbuminuria or overt proteinuria (26–28). Additionally, tubulointerstitial lesions were observed mainly in advanced disease and might contribute to the progression to ESKD in patients with T1DM (28, 29). The pathological disparity in different types of DM may be attributed to various other diabetogenic stimuli other than high glucose, including insulin resistance and growth factors and cytokines, which activate inflammatory, apoptosis, ischemic, pro-oxidant, and fibrotic pathways. A growing number of studies have proven that genes associated with pathological features of DKD are regulated not only by classical signaling pathways but also by epigenetic mechanisms involving chromatin histone modifications, deoxyribonucleic acid (DNA) methylation, and non-coding ribonucleic acid (RNA) (30).

Functional Changes

Tubular functional changes in DKD mainly correspond to the modulation of high-glucose, oxygen metabolic disorder, inflammation, fibrosis, and apoptosis (31). **Figure 1** displays the primary mechanism of tubular damage in DKD. Hyperglycemia directly destroys renal tubular cells, resulting in a wide range of cellular and metabolic dysfunctions. Three interrelated and cardinal pathways, including overproduction of reactive oxygen species (ROS), initiation of autophagy, and activation of the apoptotic pathway, are triggered by high glucose and are associated with the progression of DKD (32, 33). Oxidative

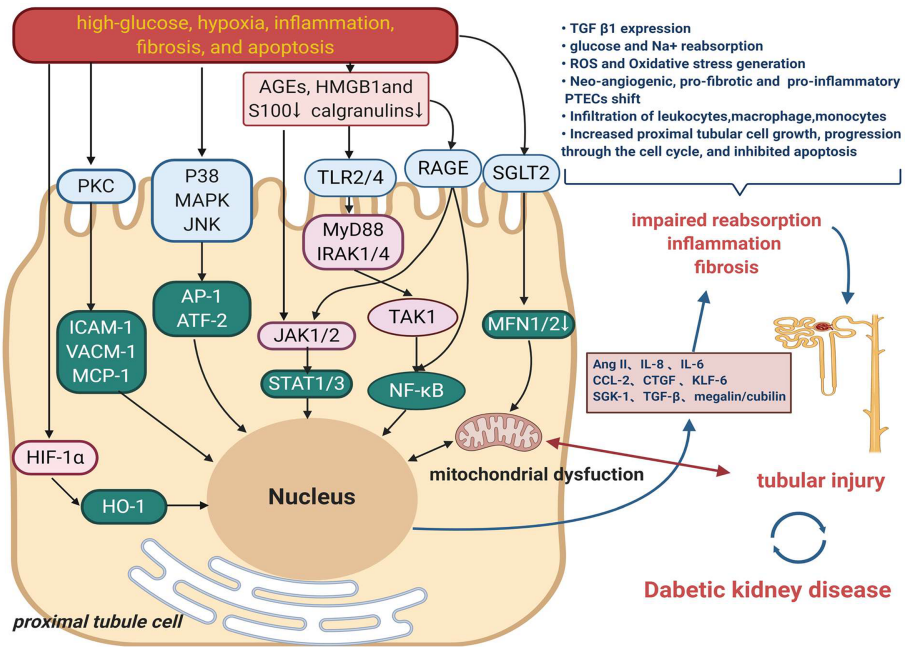

FIGURE 1 | The main mechanism of tubular damage in DKD. Diabetogenic stimuli including high-glucose, oxygen metabolic disorder, inflammation, fibrosis, and apoptosis result in a wide range of injured pathway such as MAPK, PKC signaling. High-mobility group box 1 (HMGB1), s100/calgranulins and advanced glycation end products (AGEs) are danger-associated molecular patterns (DAMPs) that activate cell surface pattern recognition receptors (PRRs), induce signaling events to promote the development of inflammation in DKD. Another mechanism that also might contribute to tubular damage is the increased renal content of HIF1-α. Multiple effects on proximal tubule ultimately result in impaired reabsorption, inflammation and fibrosis, which contribute to tubule injury and therefore DKD.

stress is a state of imbalance in the production of ROS and antioxidant activity in the body, resulting in the activation of downstream inflammation (34) and tubulointerstitial fibrosis-related genes such as transforming growth factor (TGF)-β1 and RAAS-related genes (35). Nitric oxide (NO) synthase, xanthine oxidase, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase enzymes, and the mitochondrial respiratory chain contribute to kidney ROS generation in a physiological context (36). The pro-oxidant nitrogen oxide (Nox) family members, especially Nox4 and Nox5 isoforms, have been reported to have an important role in the generation of renal ROS in diabetes. Thallas-Bonke V et al. indicated that targeted deletion of NADPH oxidase Nox4 from proximal tubules was dispensable for DKD development (36, 37).

Recent studies stress that the oxygen metabolic disorder which leads to oxidative stress, advanced glycation, hypoxia, and other harmful effects, plays a vital role in renal tubules injury (38, 39). Production and utilization of ATP by the proximal tubular cells are balanced by kidney blood flow, oxygen, and metabolite reabsorption, delivery, and consumption. This balance is now believed to the principal mechanism for regulating tubuloglomerular feedback and maintaining kidney function in diabetes (5, 33). A lately report found that hypoxia-inducible factor-1α (HIF-1α) activation in tubular cells played an important protective role against diabetic kidney injury by modulation of mitochondrial dynamics through heme oxygenase-1 (HO-1) upregulation, highlighting the potential mechanism and target in DKD (40).

Tubular inflammation is a hallmark of the progression of kidney disease in patients with DM (4). DKD inflammation produces several chemokines, which promote a pro-inflammatory microenvironment and amplify renal injury (41, 42). The majority of the pro-inflammatory responses observed in diabetic kidneys involve the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The activation of NF-κB and the transcription of certain pro-inflammatory chemokines in tubular epithelial cells are the markers of progressive DKD (43). Gene expression profiling of the tubulointerstitial compartment of patient biopsies has also identified 54 upregulated NF-κB target genes in progressive DKD (44). These studies showed that NF-κB activation stimulated macrophage recruitment and production of inflammatory cytokines [monocyte chemotactic protein-1 (MCP-1)], tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6) in diabetic kidneys, which were associated with the progression of the disease (45, 46).

In diabetic kidneys, excessive amount of plasma proteins, including albumin, filtered through the damaged glomerulus appears in the glomerular filtrate. Conventional perspectives have emphasized the role of glomerular hypertension and hyperfiltration in the early stage of DKD, which induce the increase in serum creatinine and urinary albumin excretion (47).

However, more recent studies have focused on an unchanged glomerular albumin filtration and reduced tubular albumin reabsorption (7, 48). A membrane-associated endocytic receptor megalin (low-density lipoprotein receptor-related protein 2; LRP2) drives the reabsorption of nearly all filtered plasma proteins in cooperation with the receptor protein cubilin (49–51). Protein-overloaded condition occurs in the proximal tubular epithelial cells of the diabetic kidney. Several experimental studies have indicated that protein overload induces proximal tubular cell apoptosis (52), oxidative stress (53), inflammation, and tubulointerstitial fibrosis (54–56). The clinical relevance of increased proximal reabsorption and hyperfiltration in diabetes has been demonstrated by the ability of SGLT2 inhibitors (SGLT2is) to improve renal outcomes in patients with diabetes in large-scale clinical trials, promoting the emergence of the renal tubulocentric hypothesis (15).

A Link of Diabetogenic Stimuli to Morphological and Functional Changes in Tubules

High levels of glucose-induced oxidative stress contribute to cell death in tubule injury and tubulointerstitial fibrosis in DKD (57). In addition, persistently high levels of glucose can cause abnormal activation of mitochondrial and endoplasmic reticulum stress and intracellular signal transduction pathways, leading to further activation of downstream inflammatory factors and induction of innate immune response (58). The innate immunity in native kidney cells is upregulated at the stage of diabetic microalbuminuria, while tubulointerstitial kidney cell infiltration is associated with albuminuria and fibrosis at a more advanced stage (59). Moreover, it was shown that macrophage accumulation in the interstitium, but not glomeruli, was associated with albuminuria and renal function loss (58). Clustered renal neutrophils were mostly observed in the peritubular space and were associated with accelerated progression and eventual kidney function loss (60). Mast cell accumulation and degranulation were observed in patients with T2DM at varying stages in the periglomerular, peritubular, and perivascular regions of the interstitium. Their presence correlated with tubulointerstitial injury and disease progression (61). These studies suggested that renal tubulointerstitial infiltration by inflammatory cells could accelerate tissue damage. Besides, the components of the glomerular filtrate, such as albumin, advanced glycation end products, growth hormones, *etc.*, interacted with the tubular system and contributed to increased energy consumption, renal oxidative stress, cortical interstitial inflammation, impairment of autophagy, stimulation of hypoxia, and tubulointerstitial fibrosis in DKD (6, 62–64). More convincingly, Vallon et al. illustrated that several diabetogenic stimuli (oxidative stress, tubular renin–angiotensin system, enhanced filtration, and tubular expression of growth factors) induced the growth of the proximal tubules and enhanced tubule reabsorptive capacity, resulting in inflammation, fibrosis, scarring, and impairment of renal function in the diabetic kidney (15).

CHALLENGES AND PROGRESS IN THE APPLICATION OF NOVEL TUBULAR BIOMARKERS

In clinical practice, therapeutic strategies for early identification of the kidney lesions in diabetic conditions and consequent slowing of the progression of DKD are still limited and currently mostly rely upon conventional biomarkers. The urine albumin-to-creatinine ratio (uACR) and eGFR are well-standardized and widely used biomarkers for evaluating kidney function and determining different stages of kidney disease in clinical practice. Although carrying prognostic information, eGFR is subject to variation owing to the analytical error of the creatinine measurement and biological variation derived from serum creatinine, patient's age, and gender (65, 66). ACR, a tubuloglomerular-centric marker, has been recognized as the hallmark of DKD and precedes renal function loss in years. It not only reflects the capacity of glomerular permeability but is also a valuable indicator of tubular damage or dysfunction. The increase in albuminuria followed by glomerular hyperfiltration places a burden on the proximal tubule and elicits an inflammatory response leading to tubulointerstitial damage (67). Nevertheless, a substantial proportion of patients with T1DM or T2DM have renal function impairment without proteinuria, which is known as non-proteinuric DKD (68–70). The data on clinicopathological characteristics, renal prognosis, and all-cause mortality are limited to a handful of clinical trials and longitudinal studies focused on this phenotype. In 2018, the Chronic Renal Insufficiency Cohort (CRIC) study showed that the absence of albuminuria or proteinuria was common and carried a much lower risk for ESKD, chronic kidney disease (CKD) progression, or rapid decline in eGFR than those with albuminuria or proteinuria did (71). In line with this, another propensity score-matched analysis of a nationwide, biopsy-based cohort reported that non-proteinuric DKD patients presented better-controlled blood pressure and fewer typical morphological changes. They were also at a lower risk of CKD progression and all-cause mortality (72). The possible mechanism of developing non-proteinuric DKD may rely on racial/ethnic differences, aging, and response to RAAS inhibitors or other glomerulus-protective drugs before the diagnosis of DKD (68, 73). Therefore, there is still a compelling need to discover potential novel biomarkers for early diagnosis and timely risk stratification in DKD.

Recent advancements in omics-based biomarkers including proteomics, metabolomics, genome, transcriptome, or lipidome and the integration of these different approaches continue to unveil new potential biomarkers (74). Urinary novel proteomics, peptidomics markers may be associated with impaired proximal tubular reabsorption that almost all of these filtered proteins are reabsorbed into the proximal tubules through megalin/cubilin-mediated endocytosis (75). One study also demonstrated that empagliflozin, the SGLT2i, significantly impacts urinary peptides (76). However, their detection is relatively expensive and still needs time to promote clinical use. Rigorous technical and clinical validation studies are demanded to clarify the specific

role and the underlying mechanism. Future research in DKD should attempt to explain how the novel biomarkers can be combined with traditional clinical and biochemical biomarkers in clinical practice to guide screening programs, improve risk stratification, predict response to treatment, and provide a method of monitoring response to treatment. The tubular biomarkers in DKD are summarized in **Figure 2**, which outlines three main classes of the principal tubular biomarkers that may be helpful in early detection and risk-stratification of DKD. The potential applications of these biomarkers in DKD were shown in **Table 1**.

Neutrophil Gelatinase-Associated Lipocalin

NGAL is a 24 kDa secreted glycoprotein that belongs to the lipocalin protein family. As mainly released by neutrophils and distal tubular cells, it rapidly increases when acute tubular damage of various causes occurs (109). Following the discovery that NGAL levels are also raised in the CKD setting, this marker has been suggested to correlate with CKD progression (77, 78). More importantly, a great number of studies have demonstrated the important role of NGAL in predicting the evolution of DKD. In a study of T2DM patients and healthy controls, Fu et al. reported that NGAL increased across the four groups from controls to normoalbuminuric, microalbuminuric, and macroalbuminuric patients (79). In several observational single-center follow-up studies, elevated urine NGAL level was shown to be associated with urinary albumin excretion (80), the rapid decline in eGFR and increased serum creatinine (81), renal progression to ESKD (83), and progressive tubular structural and functional impairment (84). Consistently, our cohort study found that the best predictive cutoff value of urinary NGAL to creatine ratio (uNCR) for DKD diagnosis was 60.685 ng/mg, and T2DM patients with the increased level of uNCR had a higher risk of nephrotic-range proteinuria and worse renal outcome (82). Furthermore, a more recent report from the CRIC study conducted at seven US clinical centers provided solid evidence that higher urinary NGAL levels were not only strongly associated with cardiac markers, but were also linked to an approximately twofold or greater risk of CKD progression in patients with DM (10). It has been postulated that NGAL captures some of the variability in the rate of kidney function decline independently of albuminuria or other risk factors and reflects tubular injury and inflammation in the setting of DKD (10, 85).

Kidney Injury Molecule 1

KIM-1 is a transmembrane protein expressed on the apical membrane of proximal tubule cells (110). KIM-1 facilitates the repair of the injury by removing apoptotic bodies and cellular debris from the damaged tubulointerstitial compartment (8). Han et al. reported that urinary KIM-1 was not detectable in normal kidneys while its levels were upregulated with the occurrence of kidney injury (86). Consistently, renal KIM-1

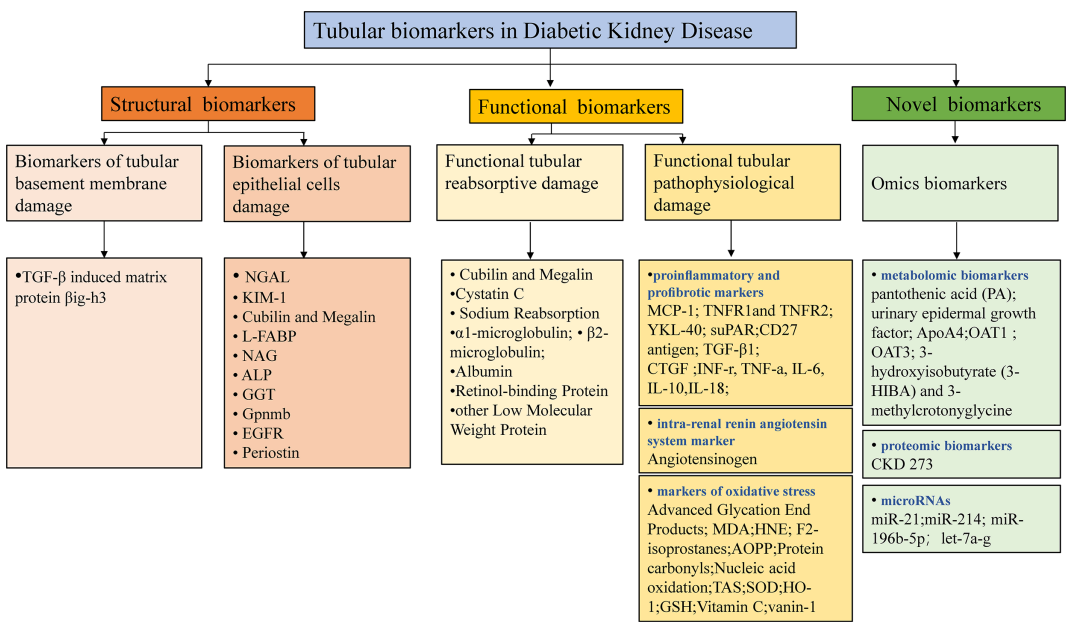

FIGURE 2 | Potential tubular biomarkers in DKD. TGF-β, transforming growth factor-β; NGAL, neutrophil gelatinase-associated apolipoprotein; KIM-1, kidney injury molecule 1; YKL-40, chitinase-3-like protein 1; MCP-1, monocyte chemoattractant protein-1; L-FABP, liver-type fatty acid binding protein; NAG, N-acetyl-β-D-glucosidase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; Gpnmb, glycoprotein Nmb; EGFR, epidermal growth factor receptor; TNFR1/2, tumor necrosis factor receptor 1/2; suPAR, soluble urokinase receptor; CTGF, connective tissue growth factor; INF-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL-6/10/18, interleukin 6/10/18; MDA, malondialdehyde; AOPP, advanced oxidation protein products; SOD, superoxide dismutase; HO-1, hemeoxygenase-1; GSH, glutathione; PA, pantothenic acid; OAT1/3, organic anion transporter1/3; 3-HIBA, 3-hydroxyisobutyrate; CKD, chronic kidney disease; TAS, total antioxidant status.

TABLE 1 | Summary of principal tubular biomarkers of DKD in clinical use.

Tubular biomarkers	Clinical Importance	Sample	Ref.
NGAL	increased when acute tubular damage of various causes occurred; correlated with CKD progression	urine	(77, 78)
	associated with urinary albumin excretion, rapid decline of eGFR, and increased serum creatinine	urine	(79–82)
	associated with renal progression to ESKD, progressive tubular structural and functional impairment	urine	(10, 83–85)
	best predictive cutoff value of urinary NGAL to creatine ratio (uNCR) for T2DKD diagnosis was 60.685 ng/mg; 7.595 times higher risk of nephrotic-range proteinuria in T2DKD patients with uNCR >60.685 *vs.*≤60.685 ng/mg.	urine	(82)
	twofold or greater risk for CKD progression in patients with diabetes; 1.5-fold or greater risk for CKD progression in patients without diabetes	urine	(10)
KIM-1	repaired injury by removing apoptotic bodies and cellular debris	urine	(8)
	upregulated when kidney damages	urine	(86)
	largely restricted to tubular cells in areas with tubulointerstitial damage induced by overload proteinuria; upregulated in proteinuric nephropathy and associated with renal fibrosis and inflammation.	tissue	(55) (87)
	elevated in T2DM with normal or mildly increased albuminuria	urine	(88)
	increased in T1DM patients who developed from macroalbuminuria to late-stage CKD	urine	(89)
	elevated in the high-risk group which was stratified by both ACR and eGFR; decreased in the very high-risk group; not associated with either eGFR or albuminuria	urine	(84)
	no predictive value for progression to ESKD independently of albumin excretion rate (AER); no prognostic benefit to conventional biomarkers (AER, eGFR); causal impact of KIM-1 on the decrease of eGFR in T1DM by Mendelian randomization analysis	urine	(89)
	no association with uKIM-1-to-creatinine ratio and eGFR decline in patients with T2DM	urine	(13)
	contains most of the predictive information for eGFR progression in T1DM	urine	(90)
	predictive value for the rapid decline of renal function in DKD	urine/ serum	(81, 91, 92)
	associated with DKD progression and yearly decline in eGFR	plasma	(9)
	the most important predictor by cross-omics technologies	urine	(93)
YKL-40	a marker of inflammation and endothelial dysfunction; an indicator of tubular injury severity	/	(94, 95)
	associated with albuminuria in T1DM and in early stage of nephropathy in T2DM	plasma	(96) (13, 94, 97)
	elevated among macroalbuminuric T2DM patients	urine	(98)
	not associated with eGFR decline and varying levels of baseline eGFR and albuminuria in T2DM	plasma	(99)
	a plasma marker of DKD progression	plasma	(9)
MCP-1	upregulated and expressed in the diabetic glomerular and renal tubular epithelium	urine	(100)
	correlated with the extent of interstitial inflammatory infiltrate	urine	(101, 102)
	associated with severity of proteinuria in DKD	urine	(103)
	elevation in renal tubuli contributes to renal tubular damage in DKD	tissue	(103)
	MCP-1-to-creatinine ratio concentrations were strongly associated with sustained renal decline, severity of kidney damage in T2DM	urine	(13) (84)
	associated with an increased risk of DKD progression only among patients with baseline eGFR<45 ml/min per 1.73 m^2	plasma	(9)
Cubilin and megalin	increased in microalbuminuria groups compared with non-albuminuric groups in T1DM	urine	(104)
	genetic association exists between a cubilin and a rare megalin variant with diabetes-associated ESKD in populations with recent African ancestry	gene	(105)
	upregulated renal megalin expression in early T2DM rats	tissue	(106)
	elevated in two models of insulin-deficient diabetes in drug-inducible megalin knockout mice	tissue	(107)
	megalin in both segment 1 and segment 2 participated in clearing the ultrafiltrate from proteins in both cortical and juxtamedullary nephrons under normal conditions	tissue	(108)
	megalin in segment 3 was inactive with regard to protein endocytosis; it was activated by the presence of proteins in the lumen of the tubule in normal physiology	tissue	(108)

NGAL, neutrophil gelatinase-associated apolipoprotein; KIM-1, kidney injury molecule 1; YKL-40, chitinase-3-like protein 1; MCP-1, monocyte chemoattractant protein-1; T1DM/T2DM, type 1/2 diabetes mellitus; CKD, chronic kidney disease; ESKD, end-stage kidney disease; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; AER, albumin excretion rate; ESKD, end-stage of kidney disease; uNCR urinary NGAL to creatine ratio.

expression was largely restricted to tubular cells in areas with tubulointerstitial damage in an experimental model of tubulointerstitial damage induced by overload proteinuria (55), and it was also upregulated in patients with proteinuric nephropathy (87). Hence, KIM-1 was suggested to be a specific and sensitive biomarker of proximal tubular damage. However, there has been a controversy about the changes in its serum and urine levels, as well as its association with kidney progression in DKD. In several studies, urine KIM-1 was elevated in T2DM patients with normal or mildly increased albuminuria (88) and in T1DM patients who developed from macroalbuminuria to late-stage CKD (89). However, Siddiqui et al. found that urinary KIM-1 was elevated in the high-risk group (stratified by both ACR and eGFR) and reduced in the very high-risk group. Also, it was not found to be associated with either eGFR or albuminuria (84). The disparity of those studies may be due to the limited sample sizes and selected population. In a large-sample randomized-controlled trial in T1DM conducted by Panduru et al., KIM-1 had no predictive value for progression to ESKD independently of albumin excretion rate (AER) and added no

prognostic benefit to conventional biomarkers (AER, eGFR). However, the causal impact of KIM-1 on the decrease of eGFR in T1DM was confirmed by Mendelian randomization analysis (89). Nadkarni et al. did not find any association with uKIM-1-to-creatinine ratio and eGFR decline in patients with T2DM and preserved renal function from the ACCORD Trial population (13). Another recent report in T1DM patients from the Scottish Diabetes Research Network Type 1 Bioresource (SDRNT1BIO) and the Finnish Diabetic Nephropathy (FinnDiane) study showed that just the serum KIM-1, as well as CD27, contained most of the predictive information for eGFR progression among a large set of associated biomarkers evaluated with the Luminex platform and LC electrospray tandem MS (LC-MS/MS) (90). More recent evidence still emphasizes the important role of KIM-1 in DKD. In 2020, a multicenter and prospective cohort within the CRIC Study suggested that higher plasma KIM-1 levels were associated with DKD progression and yearly decline in eGFR (9). Kammer et al. reported that the discrimination of eGFR trajectories in individuals with the incident or early DKD and maintained baseline eGFR was modest, and KIM-1 was the most critical predictor by cross-omics technologies (93).

YKL-40

YKL-40, which is composed of three N terminal amino acids tyrosine (Y), lysine (K), and leucine (L), is a low-molecular-weight (40 kDa) heparin- and chitin-binding glycoprotein. Also known as cartilage glycoprotein-39 or chitinase 3-like protein 1 (CHI3L1), YKL-40 is a product of the chitinase 3-like 1 gene and a growth factor for several cell types. It has an established role in extracellular matrix remodeling and angiogenesis (111). Moreover, YKL-40 acts as a marker of inflammation and endothelial dysfunction. It is secreted by various cells such as neutrophils and activated macrophages in different inflamed tissues and vascular smooth muscle cells (94). Increasing evidence stressed the role of YKL-40 in kidney disease. YKL-40 was demonstrated to be an indicator of tubular injury severity, and it was upregulated in kidney macrophages after ischemia–reperfusion injury (95). It played a role in limiting tubular cell apoptosis during the repair phase of acute kidney injury (AKI) (95). The association of YKL-40 with DKD has also been suggested. Several studies have suggested that urine YKL-40 has a limited role. In contrast, plasma YKL-40 was independently associated with albuminuria in T1DM and in the early stage of nephropathy in T2DM patients (13, 94, 96, 97). However, one study documented that urinary excretion of YKL-40 was significantly elevated among macroalbuminuric T2DM patients (98), while another study reported that plasma YKL-40 was not associated with eGFR decline in participants with type 2 diabetes and varying levels of baseline eGFR (mean eGFR 78 ml/min per 1.73 m^2) and albuminuria (99). More convincing results were obtained from a multicenter, prospective, large-sample cohort within the CRIC Study, providing new insights on YKL-40 as a plasma marker of DKD progression. Increased plasma YKL-40 concentrations were associated with DKD progression and decline in eGFR over time, even after adjustment for potential confounders and other plasma biomarkers (9).

Monocyte Chemoattractant Protein-1

MCP-1 (or C-C chemokine ligand 2) is a member of the C-C chemokine family, recruiting monocytes and influencing macrophage accumulation (112, 113). As an inflammatory biomarker, MCP-1 is highly upregulated in the diabetic glomerular and tubular epithelium (100). Previous studies have documented that urinary MCP-1 levels not only correlate with the extent of interstitial inflammatory infiltration but also are associated with the development of albuminuria and renal damage (101, 114). Morii et al. found that MCP-1 was produced in renal tubular cells and released into the urine in proportion to the degree of albuminuria. Increased renal tubular MCP-1 expression contributed to tubular damage in DKD (103). The ACCORD trial enrolled 10,251 T2DM patients with preserved renal function and examined the association of four biomarker-to-creatinine ratio levels; only MCP-1-to-creatinine ratio concentrations were strongly associated with the sustained renal decline (13). Siddiqui et al. also found that elevated urinary MCP-1 was related to the severity of kidney damage, and it was expressed more in progressive renal impairment in T2DM (84). The 2020 CRIC Study first reported an association of plasma MCP-1 concentrations and DKD progression among individuals with moderate to severe kidney disease. Higher plasma MCP-1 levels were associated with an increased risk of DKD progression only among patients with baseline eGFR<45 ml/min per 1.73 m^2 (9).

Cubilin and Megalin

In physiological conditions, proximal tubule epithelial cells have the capacity of reabsorbing nearly all low-molecular-weight serum proteins and ultrafiltrated albumin, along with glucose, phosphate, amino acids, and various ions. The key contributor for the uptake ability of the epithelial cells essentially relies on the collective effort of two apical membrane receptors cubilin (CUBN) and megalin (LRP2), which form a complex expressed at the brush border (115). Both cubilin and megalin are huge multiligand receptors (460 and 600 kDa, respectively), each of which could independently bind to an amount of identified substrates including albumin and vitamin D binding protein (VDBP) (49). After ligand binding, cubilin/megalin ligands interact and are internalized to proximal tubular epithelial cells' (PTECs) endosomes and lysosomes for catabolic degradation and receptor recycling (116). Using a GeLC/MS platform proteomics approach, Thrailkill et al. first propose that enhanced cubilin and megalin excretion might serve as important markers of DKD, considering that urinary cubilin and megalin were significantly higher in microalbuminuria groups than in non-albuminuric groups in T1DM patients (104). Both album infiltration and reabsorption were observed elevated in two models of insulin-deficient diabetes and drug-inducible megalin knockout mice (107). A study published in 2020 explained that megalin in both segment 1 and segment 2 participated in clearing the ultrafiltrate from proteins in both cortical and juxtamedullary nephrons under normal conditions. Although megalin in segment 3 was inactive concerning protein endocytosis, it was activated by the presence of proteins in the

lumen of the tubule in normal physiological conditions (108). These studies provided a theoretical rationale and backbone for early treatment to improve the capacity of proximal tubule to avoid the development of proteinuria.

RENAL TUBULE-TARGETING THERAPEUTICS: A NEW ERA FOR DKD MANAGEMENT

In addition to the new tubulocentric insights for DKD mentioned above, the emergence of new anti-hyperglycemic agents has considerably altered the therapeutic landscape of DKD. For decades, the cornerstone of DKD therapeutics relied on lifestyle interventions, strategies for hyperglycemia and hypertension in combination with the use of angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) (117). Recent advances in studies on novel glucose-lowering agents promote the new era in the advanced glycemic control and concurrently promise cardiorenal protection in DKD management. **Figure 3** depicts the current high-profile classes of potential novel anti-hyperglycemic agents for DKD, mainly grouped into renal tubule-targeting therapies, incretin therapies, and energy pathways-targeting therapies (117, 118). The tubule-targeting medicine, SGLT2i also affects the energy pathway associated with enhanced sirtuin1 and hypoxia-inducible factor (HIF)-2a signaling (119). In addition to SGLT2i, incretin drugs include glucagon-like peptide 1 receptor (GLP1R) agonists and dipeptidyl peptidase 4 (DPP4) inhibitors, which also have the potential to improve tubulointerstitial function. GLP1R expression was detected in macrophages, endothelial cells, juxtaglomerular cells, and proximal tubules within the kidney in various animal models and human tissue (117). Endogenous GLP1R signaling exerts a natriuretic action in DKD. Direct GLP1R-stimulation induces diuresis and natriuresis by increasing GFR and inhibiting the activity of the sodium-hydrogen exchanger isoform 3 (NHE3) in the proximal tubule (120, 121). Nevertheless, DPP4 inhibitors demonstrate modest kidney-protective effects. Compared with the GLP1R agonists, they mainly attenuate albuminuria without an impact on eGFR decline. DPP4 inhibitors indirectly modulate glucose-dependent insulin secretion and suppress glucagon secretion from pancreatic α-cells by elevating endogenous GLP1 levels (122). Linagliptin, the only available DPP4 inhibitor, showed a significant improvement in albuminuria progression but not in kidney outcomes in the Cardiovascular and Renal Microvascular Outcome Study with Linagliptin (CARMELINA) trial (123). No significant placebo-adjusted changes in eGFR or albuminuria with linagliptin therapy were observed in the Modification of Albuminuria in T2D and CKD with the LINAgliptin (MARLINA-T2D^{TM}) study (124).

Among diabetic medications, SGLT2i attracts considerable attention for their pleiotropic effects on glycemic control, renal protection, cardiovascular benefits, blood pressure control, and attenuation of lipid levels. SGLT2 is a low-capacity and high-affinity glucose transporter with 1:1 Na+/glucose stoichiometry. It is located in the S1–2 segment of the proximal convoluted tubules and is responsible for reabsorption of 90% of glucose filtered through the glomerulus (125). Multiple mechanisms are explored involving the kidney protection of SGLT2 inhibition, mainly characterized into (1) attenuation of proximal tubular oxidative stress, mitochondrial morphology, modulation of key metabolism and reabsorptive proteins, pro-inflammatory and profibrotic cytokines, and improvement of tubulointerstitial fibrosis; (2) through activation of tubuloglomerular feedback to regulate glomerular hemodynamic stability and metabolic effects (126, 127). **Table 2** summarizes the underlying mechanism of kidney protection by SGLT2 inhibition in DM reported in recent years.

There is increasing evidence suggesting that SGLT2i has renal protective effects in addition to cardiovascular protection, as reported by diverse clinical trials (summarized in **Table 3**). The first clues involving the potential nephroprotection with SGLT-2

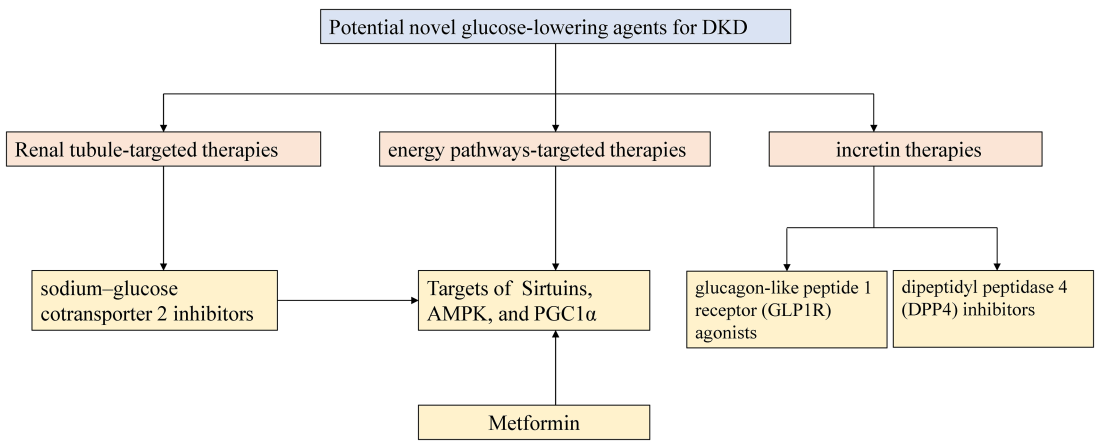

FIGURE 3 | Outlines of potential novel glucose-lowering agents for DKD. AMPK 5-AMP-activated protein kinase; PGC-1α peroxisome proliferator-activated receptor γcoactivator-1 alpha.

TABLE 2 | Proposed hypotheses for the kidney protective mechanisms of SGLT2 inhibitors in DKD.

Mechanisms	Ref.
decreased sodium uptake by Na^+/H^+ exchanger isoform 3 (NHE3) expression in proximal convoluted tubules (PTs)	(128–133)
reduced urinary excretion of angiotensin II and angiotensinogen levels in SGLT2 inhibitor-treated T2DM rats	(134)
did not further activate RAS in the long term, which prevented the RAS-mediated aggravation of cardiovascular and renal events	(134, 135)
reduced urinary angiotensinogen excretion in patients with T2DM	(136)
increased urinary angiotensinogen excretion in patients with T1DM	(137, 138)
modulated the tubular expression of proteins governing the medullary concentration activity, further had an effect on fluid and electrolyte balance	(139, 140) (132)
blocked the activation of the apoptotic-associated protein within PT cells	(141)
glomerular fibrosis or injury was not alleviated in SGLT2-knockout diabetic mice	(142)
modulated oxidative stress and intraglomerular inflammation and could thus alleviate renal fibrosis	(143)
alleviated the generation of vanin-1, the biomarker for oxidative stress within the kidney	(144)
lessened the epithelial-to-mesenchymal transition by modulating miR21	(145)
alleviated renal fibrosis by lowering lipid accumulation-induced inflammation mediated by CD68 macrophages	(146)
activation of tubuloglomerular feedback: alleviated apoptosis by increasing autophagosomal formation within glomerular mesangial cells and podocytes	(147, 148)
anti-inflammatory effects: decreased the levels of several cytokines such as tumor necrosis factorα (TNFα), interleukin-6, high-sensitivity C-reactive protein, and leptin	(149, 150)
restored oxygen supply, thereby alleviating the metabolic stress state in the mitochondria and restoring the hematocrit level in patients with DM	(151, 152)
reduced ECM fibrosis by inflammation reduction and RAAS overactivation	(153)
the EPO-producing ability in patients with DM might be reversed after treatment with SGLT2i	(154)
suppressed HIF-1α-mediated metabolic switch from lipid oxidation to glycolysis in kidney tubule cells of diabetic mice.	(155)
inhibited aberrant glycolytic metabolism and mitochondrial ROS formation in PTEC in high-glucose conditions.	(156)
via the reduction of megalin O-GlcNAcylation and the following megalin internalization and endocytic functional suppression to attenuate protein overload in renal proximal tubule in progressive DKD.	(56)
promoted elevation of ketone bodies, which subsequently inhibited mTORC1 in the proximal renal tubules, explaining their protective effects s in non-proteinuric and proteinuric DKD.	(157)
Empagliflozin protected against proximal renal tubular cell injury induced by high glucose *via* regulation of hypoxia-inducible factor 1-alpha.	(158)

NHE3, Na+/H+ exchanger isoform 3; PT, proximal convoluted tubule; SGLT2, sodium-glucose co-transporter 2; T1DM/T2DM, type 1/2 diabetes mellitus; RAS, renin-angiotensin system; RAAS, Renin-angiotensin-aldosterone System; TNFα, tumor necrosis factorα; ECM, extracellular matrix; EPO, erythropoietin; DM, diabetes mellitus; HIF-1α, hypoxia inducible factor-1α; PTEC, Proximal Tubular Epithelial Cell; DKD, Diabetic Kidney Disease; mTORC1, mammalian target of rapamycin complex 1.

inhibitors originated from glucose-lowering trials that set albuminuria as a secondary outcome (167). In the Empagliflozin Cardiovascular Outcome Event (EMPA-REG OUTCOME) trial, the treatment of empagliflozin significantly reduced the primary end points which were defined as progression to macroalbuminuria, doubling of the serum creatinine level (D-Scr), initiation of kidney replacement therapy, or renal death, and incident albuminuria (159) (**Table 3**). In addition, all individual renal end points showed notable attenuation (31, 168). In the subsequent published Canagliflozin Cardiovascular Assessment Study (CANVAS) and CANVAS-Renal (R) program studies, clear renal protective effects were also noted (160, 161). Kidney function declined in a relatively stable manner, and urine albumin loss decreased in participants who received canagliflozin *vs.* placebo. Regarding the Dapagliflozin Effect on Cardiovascular Events-Thrombolysis in Myocardial Infarction 58 (DECLARE-TIMI 58) trials, although treatment with dapagliflozin showed a non-inferior rate of major adverse cardiovascular events (MACEs) than placebo, a possible lower rate of adverse renal outcomes in the dapagliflozin group than in the placebo group was observed (163). Although the above cardiovascular trials indicated nephroprotective effects of SGLT2i, it should be noted that the recruitment of participants was biased, considering that the selected patients had a high risk of cardiovascular events and mostly normal kidney function (169). Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation(CREDENCE) was the first dedicated renal outcomes trial of an SGLT2i canagliflozin, the recruitment of which was randomized in 4,401 T2DM patients with CKD, severely elevated albuminuria, and already ACEIs or ARBs receivers (162). The incidence rates of primary composite outcomes (D-Scr, ESKD or renal/CV death) and the renal-specific composite outcomes (D-Scr, ESKD or renal death) were significantly lower in the canagliflozin group than in the placebo group. Subsequently, two trials embarked on investigating the kidney effects of SGLT-2 inhibitors in CKD patients with or without DM (169). The Dapagliflozin and Prevention of Adverse Outcomes in Chronic Kidney Disease (DAPA-CKD) trial enrolled 4,304 CKD patients with an eGFR ranging from 25 to 75 ml/min/1.73 m^2, and uACR range from 200 to 5,000 mg/g (164). The trial aimed to evaluate the effect of dapagliflozin 10 mg once daily compared with placebo in addition to a maximum tolerated labeled dose of an ACEI or ARB. Reductions of the same magnitude in the primary outcomes (a composite of a sustained decline in the estimated GFR of at least 50%, ESKD, or renal/CV death) and renal-specific composite outcomes (D-Scr, ESKD, or renal death) were noted. The benefit was comparable for patients with diabetic and non-diabetic CKD. The Heart and Kidney Protection with Empagliflozin (EMPA-KIDNEY) trial commenced in November 2018, with a plan to recruit 5,000 participants and to be completed in June 2022 (170). The empagliflozin on estimated extracellular volume, estimated plasma volume, and measured glomerular filtration rate in patients with heart failure (Empire HF Renal) trial focused on the effects of empagliflozin in both heart failure and CKD patients. It enrolled 391 patients with left ventricular ejection fraction (LVEF) ≤40% and eGFR >30 ml/

TABLE 3 | Summary of the main renal outcomes of the SGLT2 inhibitors trials.

Trial name/ drug	Study population	Primary endpoint	Renal outcomes	Effect size (SGLT2i *vs.* placebo)	Renal benefits *vs.* placebo	Ref.
the EMPA-REG OUTCOME/ empagliflozin	7,020 T2DM, established cardiovascular disease, with eGFR >30 ml/min/ 1.73 m^2	progression to macroalbuminuria D-Scr, initiation of KRT, or death from renal disease, and incident albuminuria	Doubling of Scr with eGFR ≤45 ml/min/1.73 m2, initiation of KRT, or renal death	HR 0.54 (95%CI 0.40–0.75)	Superior	(159)
			Incident or worsening nephropathy	HR 0.61(95%CI 0.53–0.70)		
the CANVAS Program/ Canagliflozin	10,142 T2DM, high cardiovascular risk, with eGFR >30 (ml/min/1.73 m^2)	a composite of death from cardiovascular causes, non-fatal myocardial infarction, or nonfatal stroke	At least 40% reduction in eGFR, need for KRT, or renal death	HR 0.60 (95%CI 0.47–0.77)	Superior	(160)
the CANVAS-R Program/ Canagliflozin	10,142 T2DM	a composite of sustained and adjudicated D-Scr, ESKD, or renal death	Progression of albuminuria	HR 0.73 (95% CI, 0.67–0.79	Superior	(161)
			D-Scr, ESKD, or renal death	HR 0.53 (95% CI 0·33–0·84)		
			40% reduction in eGFR, ESKD, or death from renal causes	HR 0.60 (95% CI 0·47–0·77)		
the CREDENCE Trial/ Canagliflozin	4,401 T2DM and albuminuric CKD	D-Scr, ESKD, or renal/CV death	D-Scr, ESKD, or renal/CV death	HR 0.70 (95% CI, 0.59–0.82)	Superior	(162)
			D-Scr, ESKD, or renal death	HR 0.66 (95% CI, 0.53–0.81)		
the DECLARE-TIMI 58/ Dapagliflozin	17,160 T2DM	MACE and a composite of cardiovascular death or hospitalization for heart failure	At least 40% reduction in eGFR to less than 60 ml/ min per 1.73 m^2, ESKD, or renal/CV death	HR 0.76 (95% CI 0.67–0.87)	Superior	(163)
			At least 40% reduction in eGFR to less than 60 ml/ min per 1.73 m^2, ESKD, or renal death	HR 0.53 (95% CI 0.43–0.66)		
DAPD-CKD	4304 CKD, with eGFR25-75(ml/min/1.73 m^2), uACR 200 to 5,000 mg/g	a composite of a sustained decline in the estimated GFR of at least 50%, ESKD, or renal/CV death	Primary outcome	HR 0.61 (95% CI 0.51–0.72)	Superior	(164)
			Renal-specific composite outcome (D-SCr, ESKD, or renal death)	HR 0.56 (95% CI, 0.45–0.68)		
Empire HF Renal trial/ Empagliflozin	391 heart failure patients, LVEF <=40%, with eGFR >30(ml/min/1.73 m^2)	the between-group difference in the changes in estimated extracellular volume, estimated plasma volume, and measured GFR from baseline to 12 weeks.	Primary outcomes	reductions in estimated extracellular volume (adjusted mean difference –0.12 L, 95% CI –0.18 to –0.05; p = 0.00056), estimated plasma volume (–7.3%, –10.3 to –4.3; p < 0·0001), and measured GFR (–7.5 ml/min, –11.2 to –3.8; p = 0.00010)	Superior in Fluid volume changes	(165)
VERTIS CV trial/ ertugliflozin	8,246 patients with type 2 diabetes and established atherosclerotic cardiovascular disease	a composite of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke (*i.e.*, a major adverse cardiovascular event).	renal-specific composite outcome (D-SCr, ESKD, or renal death)	HR 0.81 (95.8% CI, 0.63 to 1.04)	No significant benefit	(166)

D-Scr, doubling of the serum creatinine level; KRT, kidney replacement therapy; ESKD, end-stage of kidney disease; LVEF, left ventricular ejection fraction; MACEs, major adverse cardiovascular events defined as cardiovascular death, myocardial infarction, or ischemic stroke; uACR, urinary albumin-to-creatinine ratio (with albumin measured in milligrams and creatinine measured in grams); HR, hazard ratio.

min/1.73 m^2. The results showed that empagliflozin reduced estimated extracellular volume, estimated plasma volume, and measured GFR after 12 weeks, implying that fluid volume changes might be an important mechanism underlying the beneficial clinical effects of SGLT2i (165). However, the recent Evaluation of Ertugliflozin Efficacy and Safety Cardiovascular Outcomes Trial (VERTIS CV) reported no significant benefit of ertugliflozin for the renal composite outcomes (death from renal causes, renal replacement therapy, or D-Scr) (166). Further analyses in the trial using renal different end points are underway and may give more clues. To sum up, both in the cardiovascular outcomes trials, which set different definitions of renal outcomes as secondary end points, and in the dedicated trials in CKD patients in which cardiorenal composite outcomes were primary end points, SGLT2i mostly displayed a convincing significant hindering of kidney progression.

These impressive clinical trials and mechanistic studies of SGLT2i promoted the clinical guidelines and recommendations to update the optimal approaches for the prevention and management of DKD. In 2019, the American Diabetes Association (ADA), European Association for the Study of Diabetes (EASD), and European Society of Cardiology (ESC) published updated recommendations for the management of patients with T2DM and a high cardiovascular risk, highlighting the cardiorenal benefits of SGLT2i and glucagon-like peptide-1 receptor agonists (GLP-1 RA) (171–174). The ESC guidelines suggest that SGLT2i or GLP1 receptor agonists should have priority when patients coexist with cardiovascular disease and those at high or very high cardiovascular risk. Likewise, the ADA-EASD consensus report indicates that patients at high risk of cardiorenal disease are recommended to be treated with SGLT2i or GLP1 receptor agonists, independent of glycosylated hemoglobin (HbA1c) levels. Additionally, SGLT2i, as well as metformin, was recommended as first-line glycemic management for patients with T2D and CKD according to the 2020 Kidney Disease Improving Global Outcomes (KDIGO) guideline for diabetes management in CKD, in light of the kidney benefits for most patients with eGFR ≥30 ml/min per 1.73 m^2 (175). Empagliflozin and canagliflozin are FDA-approved for use in patients with eGFR ≥45 ml/min/1.73 m^2, and ertugliflozin and dapagliflozin are used for those with eGFR ≥60 ml/min/1.73 m^2 (166, 176).

FUTURE PERSPECTIVES

Great research progress in understanding the pathogenesis of tubular damage and novel biomarkers and treatments has been made, promoting us the transition into a new era of personalized diagnosis and therapy in DKD. As a complex and major complication of metabolism disease, diabetic tubular dysfunction should be regarded with close interconnection with glomerular changes and compact interrelation with systemic metabolic changes. The major current challenges in discovered biomarkers in DKD include the integration of clinical and biochemical biomarkers and omic biomarkers and translation into the pathophysiology, differential diagnosis, risk stratification, prognosis, and individual therapy in clinical practice. The ongoing progress with new anti-hyperglycemic agents provides invaluable and novel insights into the pathophysiology and potential biomarkers of renal tubules in DKD, the combination of which will shed light on better clinical management of DKD.

AUTHOR CONTRIBUTIONS

SD drafted the manuscript, designed the figures and tables. FL and DS corrected the figures and tables. CZ and BZ reviewed the draft. CX was responsible for the final substance. YY was the guarantor and supervised the review and edited the review. All authors contributed to the article and approved the submitted version.

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Global, Regional, and National Burden of Diabetes-Related Chronic Kidney Disease From 1990 to 2019

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Background: Chronic kidney disease (CKD) is a public health problem largely caused by diabetes. The epidemiology of diabetes mellitus–related CKD (CKD-DM) could provide specific support to lessen global, regional, and national CKD burden.

Methods: Data were derived from the GBD 2019 study, including four measures and age-standardized rates (ASRs). Estimated annual percentage changes and 95% CIs were calculated to evaluate the variation trend of ASRs.

Results: Diabetes caused the majority of new cases and patients with CKD in all regions. All ASRs for type 2 diabetes–related CKD increased over 30 years. Asia and Middle socio-demographic index (SDI) quintile always carried the heaviest burden of CKD-DM. Diabetes type 2 became the second leading cause of CKD and CKD-related death and the third leading cause of CKD-related DALYs in 2019. Type 2 diabetes–related CKD accounted for most of the CKD-DM disease burden. There were 2.62 million incident cases, 134.58 million patients, 405.99 thousand deaths, and 13.09 million disability-adjusted life-years (DALYs) of CKD-DM worldwide in 2019. Age-standardized incidence (ASIR) and prevalence rate (ASPR) of type 1 diabetes–related CKD increased, whereas age-standardized death rate (ASDR) and DALY rate decreased for females and increased for males. In high SDI quintile, ASIR and ASPR of type 1 diabetes–related CKD remained the highest, with the slowest increase, whereas the ASDR and age-standardized DALY rate remained the lowest there. In high SDI quintile, ASIR of type 2 diabetes–related CKD was the highest, with the lowest increasing rate. In addition, type 2 diabetes–related CKD occurred most in people aged 80-plus years worldwide. The main age of type 2 diabetes–related CKD patients was 55–64 years in Asia and Africa. The prevalence, mortality, and DALY rate of type 2 diabetes–related CKD increased with age. As for incidence, there was a peak at 80 years, and after age of 80, the incidence declined. CKD-DM-related anemia was mainly in mild to moderate grade.

Conclusions: Increasing burden of CKD-DM varied among regions and countries. Prevention and treatment measures should be strengthened according to CKD-DM epidemiology, especially in middle SDI quintile and Asia.

Keywords: diabetes-related chronic kidney disease, mortality, disability-adjusted life-years, incidence, prevalence

INTRODUCTION

Chronic kidney disease (CKD) remains a public health problem (1), which increases and affects over 75 million people worldwide (2, 3). At present, people suffer from CKD more than osteoarthritis, diabetes, or depression (4). CKD is ranked as the 12th leading cause of mortality (5) and was listed in 2013 as one of the top 10 causes of reduced life expectancy or disability-adjusted life-years (DALYs) (3). The burden of kidney disease varies greatly across the world, as does its testing and treatment (6, 7).

The most common causes of increased CKD burden are diabetes and hypertension. Diabetic nephropathy, the leading cause of end-stage renal disease (ESRD), is associated with the excess mortality in diabetic patients (8, 9). Moreover, diabetic CKD increased kidney disease–associated disability (10, 11) and triggered arterial disease and cardiovascular complications (12). Type 2 diabetes is gradually replacing infectious diseases as the main cause of CKD in less economically advanced countries, thereby causing competition for scarce medical resources (9). In patients with type 2 diabetes and mild/moderate CKD, use of metformin is associated with a significant reduction in all-cause mortality (13). In addition, the incidence of CKD caused by diabetes (CKD-DM) is determined by socioeconomic, cultural, and political factors, which have led to gaps in the current status of CKD prevention and management capabilities in countries around the world (14). Understanding the burden of CKD-DM in various countries and implementing early detection and management are important steps towards achieving equal kidney health.

Owing to broad array of data sources and scientific statistical modeling approaches (15, 16), GBD study can provide comprehensive estimates of CKD-DM burden to date. GBD 2019 study includes 369 diseases and injuries data in 204 countries and territories (4, 10). In this study, we aimed to investigate CKD-DM epidemiology and its variation trend at the global, regional, and national levels among different sex, age, and socio-demographic index (SDI). In this study, we provided a wide range of latest CKD-DM data, including incidence, prevalence, deaths, DALYs, and sequala among two sexes, four world regions, 21 regions, and 15 age-groups. These findings could provide specific guidance for decision-making and focus efforts toward the burden of inequities in CKD.

Abbreviations: ASIR, age-standardized incidence rate; ASPR, age-standardized prevalence rate; ASDR, age-standardized death rate; CKD-DM, chronic kidney disease caused by diabetes; DALY, disability adjusted life-year; EAPC, estimated annual percentage change; SDI, socio-demographic index; UI, uncertainty interval; YLD, years lived with disability.

MATERIALS AND METHODS

Study Population and Data Collection

We evaluated the CKD-DM burden (incidence, prevalence, deaths, and DALYs) and impairment (prevalence and YLDs) in 204 countries and territories within four world regions and 21 specific regions between 1990 and 2019 (**Appendix in Supplements**). All the data were retrieved using the Global Health Data Exchange (GHDx) query tool (http://ghdx.healthdata.org/gbd-results-tool). Four measures, age-standardized rates (ASRs), and impairment data of type 1 diabetes–related CKD (CKD-T1DM) and type 2 diabetes–related CKD (CKD-T2DM) were collected among different age-groups and gender. The age range included in this study was >10 years old and was segmented into 15 age-groups. Anemia (17), an impairment related to CKD-DM, was classified into three grades: mild, moderate, and severe.

SDI, ranging from 0 to 1, is a comprehensive measure of development and is an indicator of the overall fertility rate of women under 25 years of age, educational attainment, and lagging per capita income distribution in a country. Based on SDI values in 2019, countries and territories were classified into five categories: high, high-middle, middle, low-middle, and low.

Statistical Analysis

All measures (counts, rates, and ASRs) were listed with a 95% uncertainty interval (UI). All rates in this study were reported per 100,000 individuals. We calculated estimated annual percentage changes (EAPCs) and their 95% CI to estimate the trend of ASRs, with the methods having been previously described (18, 19). When the EAPC and lower CI limit are positive, ASR increased. In contrast, when the EAPC and upper CI limit are negative, ASR decreased. The DisMod-MR 2.1 model, a Bayesian meta-regression method, was used for each measure. This study was approved by the Ethics Committee of the Second Affiliated Hospital, College of Medicine, Xi'an Jiaotong University. The access to and use of GBD study data did not require informed patient consent. This study followed the Guidelines for Accurate and Transparent Health Estimates (GATHER) Reporting guideline.

RESULTS

Global Findings

In 2019, diabetes and CKD have become the seventh largest non-communicable diseases, the fourth leading cause of death, and the sixth leading cause of disability worldwide (**Figure S1**). CKD-T1DM was responsible for 12.9 thousand incident cases, 5.02 million patients, 8.20 thousand deaths, and 3.22 million DALYs

in 2019, which increased by 75.09, 88.41, 89.73, and 72.63%, respectively, over 30 years worldwide (**Table S1**). Additionally, CKD-T2DM was associated with 2.5 million incident cases, 129.56 million patients, 405.99 thousand deaths, and 9.87 million DALYs, which increased by 156.49, 94.78, 172.39, and 141.73%, respectively (**Table 1**). Type 2 diabetes has become the second leading cause of CKD and CKD–related deaths and the third leading cause of CKD related DALYs in 2019 (**Figure S2**).

As for the variation of ASRs, both age-standardized incidence rate (ASIR) and prevalence rate (ASPR) of CKD-T1DM exhibited upward trends in both genders globally (ASIR: EAPC = 1.21, 95% CI: 1.08–1.35; ASPR: EAPC = 1.15, 95% CI: 0.10–1.31). Interestingly, age-standardized death rate (ASDR) and DALY rate remained stable over 30 years (ASDR: EAPC = 0.08, 95% CI: −0.02–0.19; DALY: EAPC = −0.08, 95% CI: −0.18–0.02), falling for women but rising for men (**Table S2**). All ASRs of CKD-T2DM increased among women and men worldwide (**Table 2**). Further analysis indicated that incidence, prevalence, mortality of CKD-T1DM remained stable in all age-groups and gender. However, DALY rate showed a peak at 40–59 years (**Figure 1**). As for CKD-T2DM, the prevalence, mortality, and DALY rate increased with age. In four world regions, CKD-T2DM occurred mostly in people aged 80-plus years (**Figure 2**). The main age at which people develop CKD-T2DM, deaths, and DALYs is presented in **Figures S3**-**5**.

SDI Findings

From 1990 to 2019, middle SDI quintile carried the heaviest burden of CKD-DM (**Tables 1** and **S1**). **Figure 3** shows the drift of CKD-DM among five SDI quintiles over 30 years. ASIR and ASPR of CKD-T1DM remained the highest in high SDI quintile, with the slowest increase (ASIR: EAPC = 0.90, 95% CI: 0.77–1.03; ASPR: EAPC = 0.74, 95% CI: 0.58–0.90), whereas they increased the fastest in middle SDI quintile (ASIR: EAPC = 1.53, 95% CI: 1.39–1.68; ASPR: EAPC = 1.62, 95% CI: 1.45–1.78), where they carried the highest age-standardized DALY rate (**Table S2**).

The ASIR of CKD-T2DM remained the highest in high SDI quintile, with the slowest increasing rate (EAPC = 0.25, 95% CI: 0.20–0.31). Among five SDI categories, ASIR (EAPC = 1.14, 95% CI: 1.09–1.19) and ASPR (EAPC = 0.12, 95% CI: 0.09–0.15) increased the fastest in middle SDI quintile. Only in low-middle SDI quintile did ASPR show a downward trend (EAPC = −0.08, 95% CI: −0.14–−0.01). ASDR and age-standardized DALY rate of CKD-T2DM increased the fastest in high SDI quintile (ASDR: EAPC = 1.72, 95% CI: 1.50–1.93; DALY: EAPC = 1.28, 95% CI: 1.09–1.47), especially for males (**Table 2**).

Figure 4 showed the variation of ASRs with the increase of SDI value among 21 regions. ASIR increased with the SDI value. As opposed to CKD-T1DM, ASPR of CKD-T2DM rose before SDI value of 0.5 and then began to decline again. As for ASDR and DALY, they had two turning points with SDI value of 0.6 and 0.8.

Regional Findings

Asia carried the heaviest burden of CKD-DM, especially in South and East (**Tables 1** and **S1**). The region with the highest ASIR of CKD-T1DM changed from High-income North America in 1990 (ASIR: 2.34, 95% UI:1.99–2.73; ASPR: 104.38, 95% UI: 92.23–117.03) to Eastern Europe in 2019 (ASIR: 3.24, 95% UI: 2.70–3.87; ASPR: 156.02, 95% UI: 135.02–181.61, **Table S2**). Similarly, that with the highest ASIR of CKD-T2DM changed from High-income North America in 1990 (38.80, 95% UI: 34.84–43.15) to North Africa and Middle East in 2019 (61.33, 95% UI: 56.00–67.44).

ASIR and ASPR of CKD-T1DM increased in most regions, and the fastest in Eastern Europe (ASIR: EAPC = 2.46, 95% CI: 2.17–2.75; ASPR: EAPC = 2.41, 95% CI: 2.14–2.69). Only in High-income North America, the ASIR of CKD-T1DM decreased (EAPC = −0.11, 95% CI: −0.20–−0.02), with the slowest decrease of ASPR (EAPC = 0.11, 95% CI: 0.01–0.21, **Table S2**).

ASIR of CKD-T2DM increased in all regions, except for High-income Asia Pacific (EAPC = 0.03, 95% CI: −0.01–0.08) and High-income North America (EAPC = 0.09, 95% CI: −0.01–0.18). ASPR of CKD-T2DM decreased the fastest in South Asia (EAPC = −0.20, 95% CI: −0.31–−0.09). Alarmingly, both ASDR and age-standardized DALY rate of CKD-T2DM increased most rapidly in High-income North America (ASDR: EAPC = 3.58, 95% CI: 3.15–4.01; DALY: EAPC = 2.73, 95% CI: 2.38–3.08), but decreased largely in High-income Asia Pacific (ASDR: EAPC = −1.16, 95% CI: −1.30–−1.02; DALY: EAPC = −0.87, 95% CI: −1.06–−0.68, **Table 2**).

National Findings

The detailed data of CKD-T1DM and CKD-T2DM among 204 countries and territories are presented in **Tables S3**-**6**. China carried the highest burden of CKD-DM, followed by the United States and India. From 1990 to 2019, incident cases of CKD-T1DM increased the most in France (130.28%, 95% UI: 76.26–202.92, **Table S3**).

In 2019, people in China had the lowest ASIR of CKD-T1DM (0.81, 95% UI: 0.64–1.03). In addition, it increased faster in France than other countries (ASIR: EAPC = 3.00, 95% CI: 2.58–3.42). ASDR decreased most rapidly in Greece (EAPC = −4.57, 95% CI: −5.95–−3.17, **Table S4**).

Incident cases of CKD-T2DM increased in most countries and territories. The number of patients with CKD-T2DM increased most in Greenland (128.68%, 95% UI: 109.28–149.71), but decreased most in Afghanistan (−23.93%, 95% UI: −27.49–−19.84). Only in Solomon Islands, deaths and DALYs of CKD-T2DM decreased, and they grew largely in Armenia and El Salvador (**Table S5**). ASIR of CKD-T2DM increased faster in Morocco (EAPC = 2.73, 95% CI: 2.64–2.81) and Turkey (EAPC = 2.58, 95% CI: 2.37–2.79, **Table S6**).

Impairment Associated With CKD-DM

CKD-T1DM resulted in 655,237 cases of anemia in 2019 (mild: 56.62%; moderate: 40.27%; severe: 3.11%), increased by 82.32% over 30 years (**Table 3**). In addition, CKD-T2DM contributed to 566,181 cases of anemia in 2019 (mild: 62.19%; moderate: 34.51%; severe: 3.30%), increased by 138.55%. Years lived with disability (YLDs) of CKD-T1DM- and CKD-T2DM-related anemia grew by 56.61 and 102.88%, respectively (**Table S7**).

ASPR of CKD-T1DM-related anemia decreased only in East Asia (EAPC = −0.92, 95% CI: −1.06–−0.77), but increased the fastest in Eastern Europe (EAPC = 2.51, 95% CI: 2.16–2.85,

TABLE 1 | The global and regional burden of chronic kidney disease caused by diabetes mellitus type 2.

Location	Sex	Incident cases (No. ×1000) (95%UI)		Prevalent cases (No. ×1000) (95%UI)		Deaths (No. ×1000) (95%UI)		DALYs (No. ×1000) (95%UI)	
		1990	2019	1990	2019	1990	2019	1990	2019
Global	Both	975.17(881.61-1077.11)	2501.25(2279.95-2740.78)	66515.19(60763.91-72748.74)	129560.07(119058.25-140047.7)	149.05(119.54-179.27)	405.99(328.43-484.98)	4083.28(3296.98-4859.14)	9870.47(8114.78-11736.44)
	Female	501.35(453.54-553.86)	1231.07(1124.19-1345.36)	34280.02(31266.23-37584.92)	65757(60532.52-71003.17)	73.33(59.22-88.53)	200.52(161.86-242.49)	1972.76(1601.93-2330.19)	4699.38(3859.13-5533.42)
	Male	473.82(427.82-524.2)	1270.18(1153.23-1393.48)	32235.17(29380.16-35247.29)	63803.08(58520.06-69067.59)	75.72(60.07-92.34)	205.47(165.23-247.34)	2110.52(1667.64-2545.21)	5171.09(4183.01-6233.02)
Socio-demographic index									
High SDI	Both	359.06(324.69-397.2)	710.94(648.66-775.19)	12128.36(11230.29-13033.34)	20966.26(19592.28-22376.84)	25.1(19.96-30.87)	79.74(61.62-99.49)	607.35(503.48-719.08)	1525(1252.92-1803.41)
	Female	194.22(175.53-214.66)	348.39(317.68-380.09)	6506.58(6034.87-6980.88)	10702.68(9990.61-11414.09)	14.2(11.18-17.66)	43.18(32.43-54.08)	324.78(269.52-386.02)	761.65(621.46-901.87)
	Male	164.84(148.36-182.57)	362.55(329.16-396.61)	5621.78(5194.52-6056.07)	10263.58(9569.04-10980.83)	10.9(8.69-13.39)	36.56(28.49-45.57)	282.56(231.46-335.16)	763.34(622.06-909.67)
High-middle SDI	Both	228.81(205.59-253.75)	557.41(505.53-612.61)	17088.34(15547.74-18733.64)	29513.21(27085.29-31891.68)	28.45(22.86-34.44)	62.02(49.89-75.24)	773.61(619.61-915.55)	1495.23(1229.3-1766.94)
	Female	122.3(109.98-135.67)	283.26(257.03-311.13)	9205.8(8393.29-10109.68)	15592.18(14302.68-16860.54)	14.26(11.44-17.38)	31.8(25.29-38.86)	380(308.84-450.27)	735.4(607.6-869.75)
	Male	106.51(95.43-118.11)	274.15(247.81-301.35)	7882.54(7184.33-8645.36)	13921.02(12746.11-15075.49)	14.19(11.24-17.36)	30.22(24.38-36.78)	393.61(311.77-472.32)	759.83(624.02-909.52)
Low SDI	Both	37.22(33.4-41.59)	100.53(90.57-111.68)	3786.14(3421.47-4195.79)	8498.37(7718.03-9331.47)	13.3(9.98-16.47)	28.93(22.62-35.57)	350.6(268.47-435.29)	754.94(587.97-928.13)
	Female	16.72(15.02-18.73)	49.8(44.83-55.2)	1820.41(1640.21-2026.23)	4111.38(3749.58-4498.82)	5.57(4.11-7.1)	13.12(10.15-16.25)	151.49(113.16-190.22)	345.36(269.94-429.53)
	Male	20.5(18.34-22.92)	50.73(45.55-56.35)	1965.73(1774.44-2177.77)	4386.99(3976.13-4834.14)	7.74(5.72-9.77)	15.8(12-19.79)	199.11(148.38-248.67)	409.58(313.34-509.5)
Low-middle SDI	Both	117.55(105.34-131.11)	347.55(313.72-384.69)	11700.33(10543.17-12982.04)	24407.41(22284.9-26589.21)	28.95(22.06-35.96)	79.2(61.95-96.51)	835.98(648.63-1026.23)	2104.04(1648.65-2571.91)
	Female	52.31(46.88-58.48)	165.09(148.68-182.69)	5566.86(5009.5-6203.64)	11825.13(10815.17-12817.21)	12.53(9.5-15.68)	36.18(27.75-44.19)	365.91(285.2-455.46)	948.6(752.73-1152.68)
	Male	65.24(58.21-73.09)	182.46(164.27-202.96)	6133.47(5552.86-6802.53)	12582.28(11477.75-13754.45)	16.42(12.31-21.05)	43.02(32.9-53.8)	470.08(355.91-596.68)	1155.44(887.1-1446.44)
Middle SDI	Both	232.05(207.35-259.87)	748.73(680.43-820.32)	21779.21(19643.7-24126.38)	46105(42148.52-50013.89)	53.15(43.11-63.02)	155.84(126.59-185.47)	1512.98(1213.59-1800.8)	3984.29(3268.66-4743.62)
	Female	115.54(103.17-129.15)	383.81(349.73-420.78)	11163.8(10055.93-12438.78)	23491.01(21509.14-25462.71)	26.73(21.71-31.88)	76.11(61.78-91.3)	749.27(603.7-894.02)	1905.01(1553.57-2255.29)
	Male	116.51(104.14-130.7)	364.91(330.24-402.1)	10615.41(9579.45-11733.26)	22614(20661.74-24521.16)	26.42(21.17-31.92)	79.73(64.38-95.83)	763.71(602.78-921.29)	2079.27(1676.95-2498.64)
Region									
Africa	Both	56.42(62.89-50.79)	194.97(216.65-176.19)	4066.44(4453.6-3693.69)	9929.63(10751.36-9071.61)	18.91(23.3-14.36)	42.68(53.14-32.82)	465.17(572.22-357.18)	1044.81(1285.39-810.78)
	Female	29.02(32.44-26)	95.19(105.79-85.93)	2080.58(2288.36-1889.57)	5005.81(5416.61-4583.23)	8.95(11.12-6.78)	20.93(26.03-15.98)	223.58(274.12-172.88)	510.66(634.56-393.77)
	Male	27.4(30.51-24.66)	99.78(110.83-89.85)	1985.86(2171.63-1800.35)	4923.82(5358.23-4506.43)	9.97(12.53-7.49)	21.75(27.48-16.58)	241.59(300.3-184.07)	534.15(671.31-409.28)
America	Both	215.37(239.37-194.09)	537.18(587.97-490.17)	8636.25(9262.04-7977.4)	18428.64(19681.15-17173)	20.09(24.52-15.97)	89.55(107.9-71.74)	533.44(631.65-430.33)	2026.44(2412.62-1647.08)
	Female	113.31(125.13-102.45)	270.9(296.04-247.8)	4605.25(4936.99-4255.85)	9533.55(10187.78-8881.42)	10.58(13.03-8.38)	45.44(54.93-36.5)	272.73(322.28-220.86)	993.07(1186.56-814.61)
	Male	102.06(114.12-91.23)	266.28(292.35-242.09)	4030.99(4323.97-3721.44)	8895.09(9515.83-8278.45)	9.51(11.59-7.48)	44.1(53.44-34.85)	260.72(309.71-208.79)	1033.37(1241.41-833.43)
Asia	Both	449.36(499.4-404.34)	1292.03(1418.95-1169.83)	41362.54(45796.5-37448.97)	83892.12(91100.56-76589.75)	91.82(109.7-74.3)	235.33(279.84-191.47)	2644.54(3142.28-2122.98)	6075.01(7221.28-4960.95)
	Female	210.93(234.32-189.26)	615.98(676.64-558.96)	20783.2(23149.92-18742.64)	42104.75(45602.97-38433.06)	43.69(52.08-35.33)	112.05(134.34-90.19)	1240.87(1481.96-992.52)	2811.43(3333.34-2302.49)
	Male	238.43(265.49-214.95)	676.05(744.83-611.18)	20579.34(22730.62-18644.22)	41787.38(45542.12-38207.77)	48.12(58.86-37.99)	123.27(148.76-98.87)	1403.68(1694.39-1100.63)	3263.59(4002.04-2634.77)
Europe	Both	252.41(278.24-227.39)	472.57(515.61-431.49)	12356(13362.41-11404.76)	17125.45(18338.75-15943.6)	17.82(22.72-13.55)	37.46(48.89-27.46)	430.21(528.12-339.33)	702.63(861.49-558.14)
	Female	147.28(162.58-132.79)	246.77(269.16-224.91)	6763.59(7308.31-6262.98)	9020.59(9624.32-8412.57)	9.91(12.71-7.51)	21.62(28.78-15.62)	231.07(282.47-182.43)	374.06(458.96-298)
	Male	105.13(116.72-94.17)	225.8(245.8-205.7)	5592.41(6063.73-5139.49)	8104.85(8708.2-7520.43)	7.9(10.2-6)	15.85(20.84-11.93)	199.15(246.65-155.04)	328.57(406.66-258.02)
Andean Latin America	Both	4.04(3.65-4.52)	21.14(19.05-23.33)	334.11(281.14-402.51)	962.88(823.71-1131.26)	1.2(0.94-1.49)	5.28(3.98-6.77)	28.31(21.98-34.68)	110.23(84.08-140.91)
Australasia	Both	8.34(7.74-9.04)	19.49(17.62-21.48)	227.65(211.32-243.83)	467.45(433.92-503.36)	0.12(0.09-0.16)	0.57(0.39-0.83)	4.01(3.2-5.02)	12.75(9.62-16.43)
Caribbean	Both	5.45(4.89-6.08)	17.09(15.54-18.81)	391.01(354.87-432.69)	779.97(719.12-843.52)	1.21(0.97-1.46)	3.38(2.59-4.22)	31.69(25.28-38.22)	85.23(66.85-105.5)
Central Asia	Both	6.32(5.48-7.28)	17.52(15.27-20.08)	841.44(758.01-938.02)	1434.75(1308.45-1573.23)	1.22(0.91-1.58)	3.02(2.33-3.72)	39(30.09-48.79)	92.94(72.42-114.09)
Central Europe	Both	27.97(24.83-31.68)	61.6(55.09-68.62)	1808.73(1659.88-1970.37)	2488.48(2307.31-2677.07)	2.65(1.97-3.36)	4.12(3.02-5.46)	72.43(54.82-90.3)	100.97(76.78-127.36)
Central Latin America	Both	31.71(28.36-35.62)	128.82(117.94-139.96)	1994.18(1802.07-2211.15)	5292.85(4897.66-5697.45)	5.07(4.11-6.05)	30.39(24-37.22)	138.58(112.85-163.25)	763.21(606.4-925.52)
Central Sub-Saharan Africa	Both	2.64(2.35-2.98)	8.13(7.27-9.09)	330.93(293.01-379.47)	794.64(710.14-894.94)	1.34(0.99-1.74)	2.76(1.92-3.71)	34.97(25.59-45.31)	70.9(49.8-94.95)
East Asia	Both	172.07(152.47-193.06)	458.41(413.64-506.95)	17631.4(15827-19724.77)	32313.15(29293.79-35236.46)	29.39(23.59-35.42)	67.92(53.83-81.92)	904.82(712.05-1092.64)	1760.71(1419.11-2118.73)
Eastern Europe	Both	37.73(33.33-42.63)	65.22(57.77-73.96)	4750.71(4335.35-5188.57)	5467.98(5017.55-5927.62)	1.24(0.86-1.67)	2.15(1.51-2.93)	50.43(38.26-64.67)	73.48(55.97-94.26)
Eastern Sub-Saharan Africa	Both	8.78(7.89-9.76)	23.72(21.23-26.37)	1045.6(944.12-1149.85)	2383.87(2162.64-2613.37)	4.69(3.57-5.9)	8.91(7.04-11.04)	117.8(90.32-145.99)	213.36(167.74-261.06)
High-income Asia Pacific	Both	73.77(67.09-81.64)	169.23(153.07-185.9)	3097.73(2849.74-3359.26)	5565.4(5189.09-5942.61)	8.66(7.17-10.18)	20.33(15.45-24.95)	194.22(164.37-222.92)	339.78(282.43-396.59)
High-income North America	Both	139.29(123.77-155.14)	262.82(236.67-291.2)	4205.65(3887.71-4527.02)	7459.82(6908.89-8008.41)	6.85(5.06-9.04)	34.02(26.61-42.21)	187.26(145.44-230.87)	706.43(566.96-843.06)
North Africa and Middle East	Both	60.32(54.04-67.27)	265.94(241.66-294.37)	2800.34(2558.83-3051.41)	8178.36(7522.26-8778.6)	15.14(11.85-19.22)	34.94(26.93-43.88)	370.21(293.31-453.59)	863.7(678.14-1069.67)
Oceania	Both	0.48(0.42-0.53)	1.44(1.26-1.61)	76.08(66.76-87.25)	173.89(154.87-196.4)	0.18(0.14-0.23)	0.49(0.38-0.63)	6.11(4.69-7.63)	16.12(12.34-20.44)
South Asia	Both	113.51(101-127.35)	336.49(300.75-375.01)	12131.69(10921.7-13489.19)	26710.17(24346.73-29149.49)	25.02(17.91-32.35)	75.5(56.32-94.73)	734.25(546.15-940.75)	2053.21(1534.68-2589.37)
Southeast Asia	Both	49.85(44.64-55.49)	181.86(164.12-200.65)	6441.62(5765.08-7213.32)	13931.04(12695.85-15228.08)	21.69(17.7-25.72)	54.67(44.56-65.66)	625.79(499.38-750.46)	1475.06(1195.58-1779.95)
Southern Latin America	Both	13.17(11.7-14.64)	32.55(29.53-35.83)	497.25(453.91-538.82)	988.22(914.99-1066.04)	2.56(2.01-3.14)	6.33(4.94-7.94)	57.06(45.28-68.25)	119.6(94.7-145.44)
Southern Sub-Saharan Africa	Both	5.89(5.25-6.66)	16.92(15.3-18.83)	418.34(380-460.58)	875.76(802.24-952.59)	1.13(0.83-1.47)	3.7(2.84-4.63)	29.62(22.02-38.25)	89.63(68.71-111.86)
Tropical Latin America	Both	23.01(20.67-25.52)	78.31(71.21-86.59)	1285.14(1174.08-1403.04)	3080.38(2849.35-3306.98)	3.56(2.84-4.28)	10.92(8.76-13.2)	98.49(79.2-117.44)	258.3(210.29-307.04)
Western Europe	Both	176.26(158.42-195.24)	293.23(266.22-320.3)	5080.3(4713.54-5452.88)	7474.3(6975.82-7958.19)	10.39(7.55-13.9)	24.91(17.37-34.13)	222.1(171.72-278.1)	380.27(294.05-489.27)
Western Sub-Saharan Africa	Both	14.57(13.14-16.09)	41.31(37.22-45.69)	1125.29(1026.97-1235.53)	2736.7(2489.79-2988.72)	5.75(4.24-7.41)	11.67(8.91-14.67)	136.13(99.13-173.95)	284.6(216.36-359.54)

DALY, disability adjusted life-year; UI, uncertainty interval; SDI, socio-demographic index.

TABLE 2 | The age-standardized rates and variation trends of diabetes mellitus type 2–related chronic kidney disease.

Location	Sex	ASIR (95%UI)		EAPC (95%CI)	ASPR (95%UI)		EAPC (95%CI)	ASDR (95%UI)		EAPC (95%CI)	Age-standardized DALY rate (95%UI)		EAPC (95%CI)
		1990	2019		1990	2019		1990	2019		1990	2019	
Global	Both	24.88(22.6-27.39)	30.29(27.65-33.05)	0.65(0.63-0.66)	1526.04(1396.82-1658.25)	1576.35(1448.28-1700.21)	0.06(0.04-0.09)	4.14(3.35-4.98)	5.16(4.2-6.17)	0.92(0.79-1.06)	101.71(82.95-120.08)	120.2(99.16-142.85)	0.75(0.63-0.87)
	Female	23.53(21.35-25.89)	28.11(25.69-30.74)	0.6(0.58-0.61)	1511.42(1383.69-1647.5)	1539.3(1415.97-1662.32)	0.05(0.02-0.08)	3.63(2.95-4.39)	4.57(3.7-5.53)	0.94(0.82-1.06)	91.99(74.8-108.81)	107.8(88.51-126.96)	0.69(0.58-0.79)
	Male	26.58(24.14-29.28)	32.86(29.96-35.88)	0.68(0.66-0.7)	1548.06(1420.38-1675.76)	1619.23(1487.83-1749.47)	0.07(0.04-0.11)	4.89(3.93-5.94)	5.91(4.79-7.14)	0.83(0.68-0.97)	114.29(92-136.53)	134.5(109.41-160.69)	0.76(0.62-0.9)
Socio-demographic index													
High SDI	Both	33.61(30.48-36.95)	37.74(34.43-41.16)	0.25(0.2-0.31)	1205.47(1113.38-1293.97)	1250.65(1162.65-1341.5)	0.04(0.01-0.07)	2.38(1.91-2.91)	3.69(2.9-4.51)	1.72(1.5-1.93)	58.49(48.34-68.99)	80.85(66.99-95.28)	1.28(1.09-1.47)
	Female	31.3(28.34-34.32)	34.35(31.4-37.47)	0.21(0.16-0.26)	1164.38(1074.72-1249.21)	1209.99(1121.13-1299.26)	0.06(0.02-0.09)	2.18(1.74-2.68)	3.31(2.58-4.06)	1.64(1.41-1.86)	54.02(44.85-63.6)	72.67(60.43-85.6)	1.16(0.96-1.35)
	Male	36.85(33.42-40.58)	41.67(38-45.49)	0.23(0.17-0.3)	1267(1173-1359.65)	1303.08(1214.18-1396.37)	-0.01(-0.05-0.03)	2.73(2.19-3.37)	4.17(3.27-5.17)	1.68(1.49-1.88)	64.8(53.12-76.69)	90.37(74.26-107.3)	1.33(1.14-1.52)
High-middle SDI	Both	21.41(19.36-23.65)	27.03(24.56-29.74)	0.88(0.86-0.9)	1524.61(1394.09-1662.33)	1528.13(1398.48-1655.86)	0.02(-0.01-0.06)	2.99(2.43-3.63)	3.13(2.54-3.79)	0.34(0.17-0.51)	72.84(59.13-86.62)	73.88(61.08-87.11)	0.27(0.12-0.42)
	Female	20.04(18.12-22.2)	25.15(22.84-27.63)	0.84(0.81-0.87)	1534.9(1399.23-1680.73)	1526.26(1396.3-1655.27)	0.03(-0.01-0.07)	2.51(2.02-3.04)	2.73(2.17-3.33)	0.43(0.27-0.58)	63.7(52.01-75.22)	65.99(54.35-78.06)	0.3(0.16-0.44)
	Male	23.59(21.24-26.05)	29.54(26.84-32.46)	0.87(0.84-0.9)	1523.6(1396.24-1654.39)	1535.61(1410.33-1663.21)	0.01(-0.03-0.04)	3.84(3.06-4.73)	3.75(3.02-4.61)	0.13(-0.06-0.32)	86.51(69.61-103.39)	84.11(69.8-100.32)	0.16(0-0.32)
Low SDI	Both	15.65(14.2-17.3)	19.61(17.7-21.68)	0.93(0.87-0.99)	1275.18(1167.68-1387.65)	1325.6(1218.77-1433.87)	0.01(-0.04-0.06)	6.9(5.27-8.63)	6.69(5.26-8.16)	-0.12(-0.19–0.05)	149.58(115.74-182.93)	146.36(115.93-178)	-0.07(-0.15-0)
	Female	14.26(12.87-15.86)	19.01(17.09-21.04)	0.99(0.96-1.01)	1234.51(1131.55-1347.02)	1273.82(1173.66-1373.46)	0.01(-0.04-0.06)	5.8(4.35-7.58)	5.88(4.62-7.26)	0.04(0-0.08)	129.35(98.11-162.2)	130.76(103.2-160.77)	0.03(-0.01-0.07)
	Male	16.98(15.38-18.83)	20.24(18.29-22.36)	0.9(0.8-1)	1314.47(1202.12-1433.01)	1378.76(1264.97-1494.27)	0.02(-0.03-0.07)	8.02(6.01-10.34)	7.58(5.82-9.47)	-0.19(-0.3–0.08)	169.42(128.6-211.12)	162.94(126.37-201.68)	-0.12(-0.23–0.01)
Low-middle SDI	Both	19.17(17.32-21.27)	25.18(22.81-27.8)	0.83(0.78-0.88)	1589.04(1455.16-1735.41)	1626.8(1495.56-1764.1)	-0.08(-0.14–0.01)	5.64(4.43-6.94)	6.41(5.07-7.8)	0.45(0.28-0.62)	135.5(106.29-165.68)	152.04(119.82-184.33)	0.46(0.29-0.62)
	Female	17.28(15.57-19.24)	23.06(20.85-25.45)	0.91(0.87-0.95)	1530.34(1396.91-1678.25)	1542.18(1413.95-1666.62)	-0.11(-0.17–0.04)	4.9(3.77-6.01)	5.57(4.35-6.79)	0.45(0.33-0.56)	119.3(93.1-147.31)	132.46(105.6-159.37)	0.38(0.28-0.48)
	Male	21.02(18.95-23.32)	27.49(24.86-30.31)	0.8(0.73-0.87)	1645.58(1503.21-1793.96)	1715.16(1573.13-1860.8)	-0.04(-0.1-0.03)	6.41(4.87-8.26)	7.36(5.76-9.1)	0.5(0.27-0.73)	151.6(116.86-190.7)	173.3(134.47-214.64)	0.55(0.33-0.77)
Middle SDI	Both	22.36(20.07-24.89)	29.39(26.77-32.19)	1.14(1.09-1.19)	1730.5(1580.15-1892.77)	1784.84(1637.95-1928.44)	0.12(0.09-0.15)	6.21(5.13-7.38)	7.03(5.79-8.35)	0.65(0.56-0.74)	143.21(117.35-167.24)	159.63(132.14-188.19)	0.63(0.53-0.73)
	Female	21.63(19.39-24.09)	28.95(26.45-31.67)	1.07(1.03-1.12)	1761.37(1603.45-1932.91)	1769.3(1623.71-1914.5)	0.07(0.03-0.1)	5.85(4.83-7.05)	6.38(5.15-7.65)	0.49(0.4-0.58)	138.25(112.44-162.95)	146.37(120.26-173.15)	0.42(0.33-0.51)
	Male	23.28(20.9-25.89)	29.92(27.21-32.9)	1.2(1.11-1.29)	1702.54(1555.65-1852.27)	1802.96(1651.97-1949.35)	0.17(0.13-0.21)	6.71(5.47-8.04)	7.85(6.37-9.41)	0.79(0.69-0.88)	149.59(121.97-177.17)	174.55(143.29-208.71)	0.82(0.71-0.92)
Region													
Africa	Both	19.93(18.02-22.14)	31.53(28.61-34.82)	1.62(1.59-1.66)	1166.31(1075.35-1260.2)	1331.27(1229.8-1430.13)	0.42(0.4-0.44)	8.1(6.23-10.07)	8.54(6.68-10.62)	0.25(0.21-0.29)	167.24(130.39-204.11)	173.75(136.33-213.95)	0.2(0.16-0.24)
	Female	20.1(18.14-22.46)	29.44(26.75-32.6)	1.42(1.32-1.51)	1181.96(1086.14-1280.44)	1301.53(1202.73-1395.88)	0.34(0.33-0.35)	7.37(5.66-9.23)	7.96(6.14-9.99)	0.36(0.32-0.39)	156.62(121.91-192.55)	163(127.42-200.92)	0.23(0.19-0.26)
	Male	19.81(17.93-21.88)	33.84(30.62-37.4)	1.83(1.78-1.89)	1151.24(1061.29-1242.84)	1365.31(1261.02-1467.95)	0.51(0.47-0.55)	8.96(6.79-11.32)	9.2(7.02-11.64)	0.14(0.09-0.2)	179.04(136.55-222.11)	185.65(143.4-233.64)	0.18(0.13-0.23)
America	Both	35.15(31.84-38.92)	42.08(38.52-45.93)	0.51(0.45-0.56)	1365.5(1265.06-1464.74)	1507.84(1401.23-1611.33)	0.27(0.24-0.31)	3.36(2.67-4.11)	6.88(5.53-8.28)	2.65(2.31-3)	87.55(70.61-103.98)	159.55(130.3-189.7)	2.19(1.89-2.48)
	Female	33.46(30.34-36.9)	39.41(36.14-42.93)	0.45(0.39-0.51)	1343.57(1241.32-1442.57)	1461.49(1356.91-1562.6)	0.22(0.18-0.25)	3.07(2.44-3.76)	6.18(4.96-7.44)	2.55(2.16-2.94)	81.29(65.76-96.19)	144.25(118.24-171.56)	2.05(1.71-2.38)
	Male	37.32(33.59-41.43)	45.22(41.18-49.58)	0.55(0.49-0.61)	1396.83(1293.07-1499.89)	1562.78(1454.16-1672.99)	0.32(0.28-0.36)	3.77(2.99-4.6)	7.76(6.15-9.41)	2.72(2.42-3.02)	95.49(76.92-113.48)	177.54(143.75-212.34)	2.3(2.05-2.56)
Asia	Both	22.3(20.15-24.62)	26.7(24.26-29.28)	0.61(0.6-0.62)	1709.44(1559.63-1868.1)	1705.51(1560.86-1849.21)	-0.04(-0.07–0.01)	5.42(4.48-6.47)	5.34(4.38-6.35)	0.04(-0.07-0.15)	127.59(104.29-149.71)	125.92(103.65-148.98)	0.13(0.02-0.25)
	Female	20.46(18.4-22.65)	24.53(22.3-26.9)	0.66(0.65-0.67)	1715.71(1562.7-1885.73)	1683.3(1540.06-1822.49)	-0.05(-0.08–0.02)	4.89(4.02-5.82)	4.71(3.78-5.65)	-0.07(-0.16-0.01)	117.49(95.42-139.45)	112.5(91.92-132.92)	-0.01(-0.09-0.08)
	Male	24.33(22.02-26.89)	29.13(26.43-31.95)	0.58(0.55-0.6)	1707.16(1561.63-1856.81)	1731.3(1589.18-1877.52)	-0.02(-0.06-0.01)	6.13(4.97-7.44)	6.13(5-7.35)	0.13(-0.01-0.27)	139.28(112.14-166.1)	140.8(115.64-169.88)	0.25(0.1-0.39)
Europe	Both	23.82(21.51-26.15)	30.57(27.96-33.37)	0.82(0.79-0.84)	1269.7(1166.51-1373.35)	1269.95(1175.27-1366.01)	-0.1(-0.15–0.05)	1.77(1.36-2.25)	2.13(1.6-2.75)	1.09(0.96-1.22)	41.77(33.14-51.04)	44.42(35.31-54.08)	0.46(0.39-0.53)
	Female	22.95(20.75-25.17)	28.31(25.75-30.97)	0.68(0.65-0.71)	1228.51(1129.87-1329.52)	1209.49(1119.17-1301.51)	-0.13(-0.17–0.09)	1.53(1.16-1.94)	1.94(1.44-2.52)	1.29(1.14-1.44)	37.25(29.75-45.45)	40.2(32.33-48.64)	0.47(0.41-0.53)
	Male	25.47(22.92-28.04)	33.68(30.73-36.64)	0.92(0.89-0.94)	1327.69(1225.96-1433.46)	1343.9(1241.99-1447.52)	-0.08(-0.13–0.03)	2.26(1.71-2.92)	2.43(1.83-3.18)	0.65(0.53-0.77)	49.32(38.7-61.37)	50.09(39.51-61.86)	0.33(0.25-0.42)
Andean Latin America	Both	19.96(18.06-22.26)	37.98(34.1-41.96)	2.23(2.11-2.35)	1401.81(1181.45-1679.85)	1660.07(1426-1945.63)	0.53(0.51-0.56)	6.51(5.11-8.06)	9.85(7.38-12.66)	1.66(1.36-1.96)	138.91(108.25-169.77)	198.82(151.5-253.93)	1.43(1.16-1.7)
Australasia	Both	33.96(31.6-36.64)	38.4(34.75-42.31)	0.34(0.28-0.4)	978.34(907.27-1049.67)	1012.3(937-1096.36)	0.07(0.03-0.12)	0.58(0.43-0.78)	1.02(0.7-1.46)	2.58(2.18-2.98)	17.34(13.81-21.58)	25.2(19.08-32.44)	1.53(1.27-1.78)
Caribbean	Both	20.72(18.6-23.15)	32.91(29.94-36.13)	1.55(1.43-1.66)	1367.33(1247.76-1495.93)	1524.92(1405.22-1646.62)	0.33(0.31-0.35)	4.94(3.99-5.93)	6.52(5-8.15)	1.43(1.29-1.58)	121.07(97.08-146.39)	164.33(129.45-202.86)	1.44(1.33-1.54)
Central Asia	Both	13.01(11.43-14.88)	21.75(19.19-24.65)	1.89(1.71-2.07)	1574.2(1430.38-1732.96)	1693.4(1553.87-1845.1)	0.22(0.17-0.26)	2.66(1.99-3.46)	4.48(3.5-5.54)	1.73(1.3-2.15)	78.61(60.41-98.13)	117.66(93.25-142.98)	1.17(0.77-1.58)
Central Europe	Both	18.61(16.6-20.87)	28.65(25.85-31.68)	1.24(1.12-1.35)	1280.91(1173.03-1395.65)	1338.32(1232.46-1445.35)	0.05(0.01-0.08)	1.87(1.41-2.35)	1.83(1.35-2.4)	0.2(-0.05-0.44)	48.91(37.72-60.54)	47.48(36.46-59.44)	0.09(-0.07-0.24)
Central Latin America	Both	36.83(33.01-41.52)	53.46(49.17-58.15)	1.18(1.1-1.27)	1949.19(1784.98-2120.56)	2174.88(2015.47-2342.85)	0.3(0.27-0.33)	6.99(5.68-8.33)	13.25(10.48-16.16)	2.44(2.1-2.79)	164.34(135.96-192)	320.2(255.6-388.56)	2.5(2.15-2.85)
Central Sub-Saharan Africa	Both	11.78(10.63-12.97)	15.86(14.22-17.42)	1.04(0.95-1.14)	1094.58(991.23-1217.6)	1114.36(1016.32-1221.23)	0(-0.04-0.03)	7.81(5.94-9.87)	6.86(4.81-9.13)	-0.54(-0.59–0.5)	158.7(119.27-200.54)	137.93(98.29-181.44)	-0.58(-0.63–0.53)
East Asia	Both	19.76(17.64-22.04)	21.7(19.63-24.02)	0.53(0.44-0.61)	1683.22(1529.89-1856.17)	1614.67(1465.8-1768.41)	0.02(-0.08-0.12)	4.04(3.32-4.8)	3.71(2.96-4.44)	0.04(-0.1-0.19)	100.28(80.75-119.3)	86.06(69.83-102.66)	-0.08(-0.24-0.08)
Eastern Europe	Both	13.54(12.05-15.21)	19.38(17.33-21.82)	1.33(1.14-1.51)	1791.58(1632.18-1961.24)	1781.78(1633.91-1931.68)	-0.07(-0.11–0.03)	0.48(0.35-0.64)	0.61(0.44-0.84)	0.81(0.62-1.01)	18.28(13.99-23.18)	21.53(16.5-27.48)	0.46(0.33-0.6)
Eastern Sub-Saharan Africa	Both	12.01(10.9-13.23)	15.19(13.64-16.83)	0.8(0.73-0.87)	1063.77(974.74-1159.28)	1097.74(1012.52-1186.64)	0.05(0.02-0.08)	7.71(5.91-9.86)	7.12(5.64-8.87)	-0.35(-0.41–0.28)	160.33(123.68-199.27)	139.28(110.65-169.71)	-0.58(-0.65–0.5)
High-income Asia Pacific	Both	36.22(33-39.95)	38.16(34.72-41.98)	0.03(-0.01-0.08)	1536.74(1416.33-1664.2)	1486.78(1379.2-1599.84)	-0.2(-0.22–0.17)	4.86(4.02-5.73)	3.49(2.76-4.19)	-1.16(-1.3–1.02)	98.87(83.9-113.3)	73.24(61.75-85.24)	-0.87(-1.06–0.68)
High-income North America	Both	38.8(34.84-43.15)	41.61(37.62-45.89)	0.09(0-0.18)	1232.98(1136.28-1328.65)	1320.68(1220.86-1417.1)	0.18(0.13-0.22)	1.88(1.39-2.46)	5.05(3.93-6.21)	3.58(3.15-4.01)	53.76(41.87-66.08)	113.61(91.76-135.29)	2.73(2.38-3.08)
North Africa and Middle East	Both	35.05(31.66-38.83)	61.33(55.98-67.44)	2.03(1.92-2.14)	1413.38(1306.46-1525.98)	1762.51(1636.48-1893.37)	0.79(0.76-0.82)	10.67(8.3-14.01)	9.58(7.47-11.95)	-0.21(-0.3–0.13)	222.15(175.54-274.43)	205.94(162.38-253.6)	-0.13(-0.18–0.07)
Oceania	Both	15.17(13.44-16.89)	19.77(17.47-22.07)	0.77(0.71-0.83)	1839.48(1661.26-2051.21)	1870.4(1692.5-2060.51)	-0.03(-0.06-0)	7.12(5.7-8.71)	8.23(6.58-10.14)	0.34(0.14-0.53)	182.83(144.01-224.89)	206.91(164.32-256.32)	0.27(0.07-0.47)
South Asia	Both	19.13(17.16-21.38)	23.58(21.1-26.18)	0.53(0.43-0.63)	1691.3(1547.37-1846.62)	1714.22(1569.95-1864.4)	-0.2(-0.31–0.09)	5.34(3.92-6.91)	5.88(4.47-7.41)	0.23(-0.06-0.52)	127.52(95.75-161.41)	143.36(108.63-179.93)	0.45(0.19-0.71)
Southeast Asia	Both	19.14(17.27-21.2)	29.11(26.35-31.92)	1.41(1.39-1.44)	1982.97(1801.55-2179.38)	2094.35(1918.32-2276.54)	0.12(0.08-0.15)	9.57(8.03-11.28)	10.06(8.22-12.04)	0.24(0.17-0.31)	226.44(184.91-267.62)	235.7(194.17-281.24)	0.23(0.17-0.3)
Southern Latin America	Both	28.35(25.28-31.4)	38.54(34.99-42.37)	1.04(0.95-1.13)	1074.34(981.95-1160.8)	1226.92(1133.37-1327.77)	0.44(0.41-0.47)	5.86(4.61-7.2)	7.37(5.76-9.24)	0.81(0.46-1.16)	124.21(99.31-148.26)	142.69(113.09-173.69)	0.47(0.18-0.77)
Southern Sub-Saharan Africa	Both	21.47(19.23-24.08)	29.93(27.01-32.99)	1.05(0.85-1.25)	1260.86(1159.11-1368.86)	1382.43(1276.29-1490.27)	0.24(0.18-0.31)	4.74(3.53-6.23)	7.86(6.14-9.87)	2.32(1.97-2.66)	106.17(79.5-135.34)	162.32(125.88-201.31)	2.01(1.7-2.33)
Tropical Latin America	Both	24.63(22.18-27.37)	32.01(29.15-35.36)	0.83(0.76-0.9)	1202.69(1107.92-1299.73)	1255.66(1161.7-1346.01)	0.08(0.05-0.11)	4.59(3.7-5.49)	4.71(3.78-5.67)	0.17(0.08-0.26)	106.48(86.54-126.13)	106.63(87.46-126.49)	-0.1(-0.22-0.02)
Western Europe	Both	29.12(26.21-31.99)	31.65(28.77-34.47)	0.19(0.12-0.25)	946.84(875.58-1018)	938.14(871.53-1003.36)	-0.19(-0.25–0.13)	1.73(1.27-2.31)	2.13(1.52-2.87)	1.17(1.04-1.31)	37.65(29.23-46.55)	37.99(29.94-47.8)	0.2(0.14-0.25)
Western Sub-Saharan Africa	Both	16.69(15.1-18.31)	22.47(20.24-24.77)	1.13(1.03-1.22)	1056.66(971.5-1142.21)	1156.15(1065.21-1241.99)	0.29(0.26-0.31)	8.01(6-10.38)	7.8(6.06-9.8)	-0.15(-0.22–0.08)	159.76(118.81-202.51)	155.3(119.85-194.88)	-0.13(-0.2–0.06)

ASIR, age-standardized incidence rate; ASPR, age-standardized prevalence rate; ASDR, age-standardized death rate; DALY, disability adjusted life-year; EAPC, estimated annual percentage change; SDI, socio-demographic index; UI, uncertainty interval.

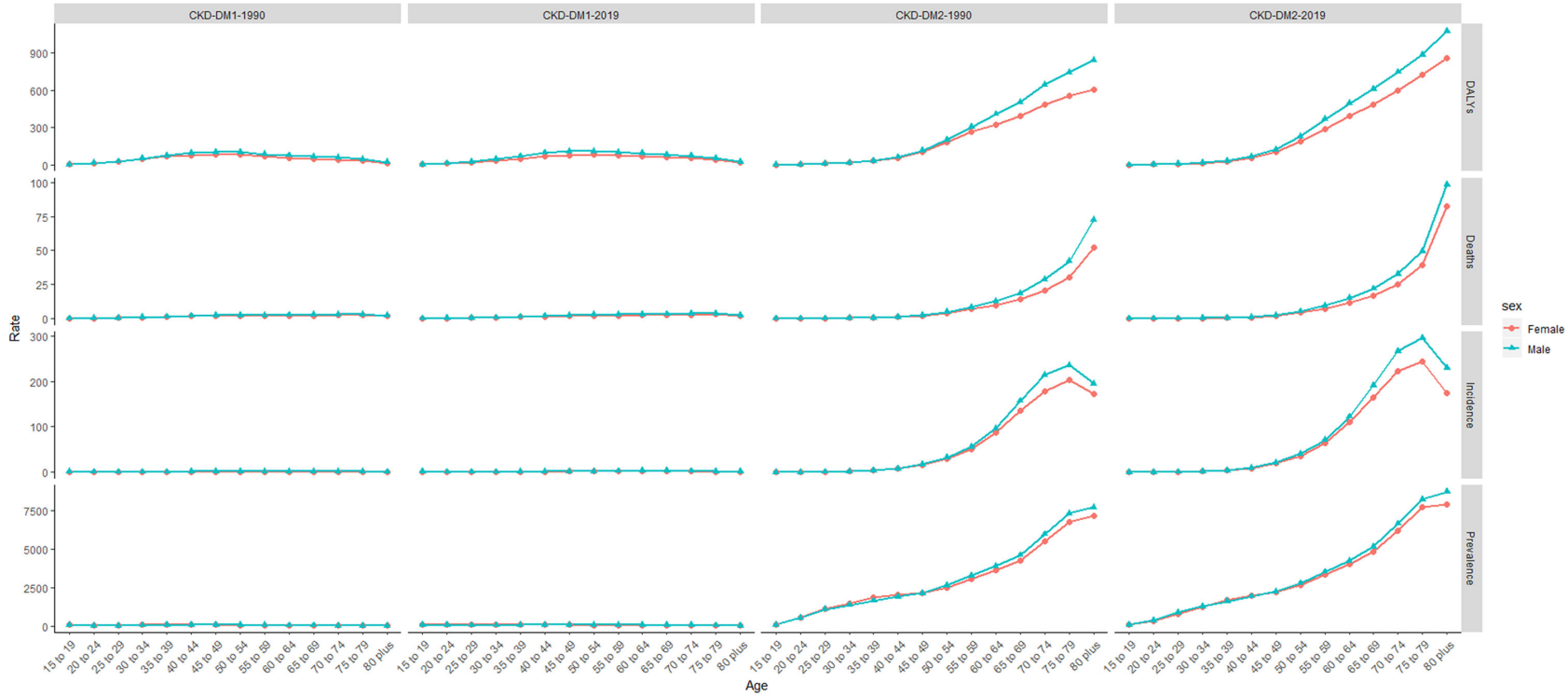

FIGURE 1 | The incidence, prevalence, death, and DALY rate of CKD-DM burden from 1990 to 2019. CKD-DM1-1990 represents the incidence, prevalence, death, and DALY rate of type 1 diabetes–related CKD in 1990. CKD-DM1-2019 represents the incidence, prevalence, death, and DALY rate of type 1 diabetes–related CKD in 2019. CKD-DM2-1990 represents the incidence, prevalence, death, and DALY rate of type 2 diabetes–related CKD in 1990. CKD-DM2-2019 represents the incidence, prevalence, death, and DALY rate of type 2 diabetes-related CKD in 2019. CKD-DM, chronic kidney disease caused by diabetes; DALY, disability adjusted life-year. The vertical axis is the incidence, prevalence, death, and DALY rate (per 100,000 people), and the horizontal axis is the different age-groups (years).

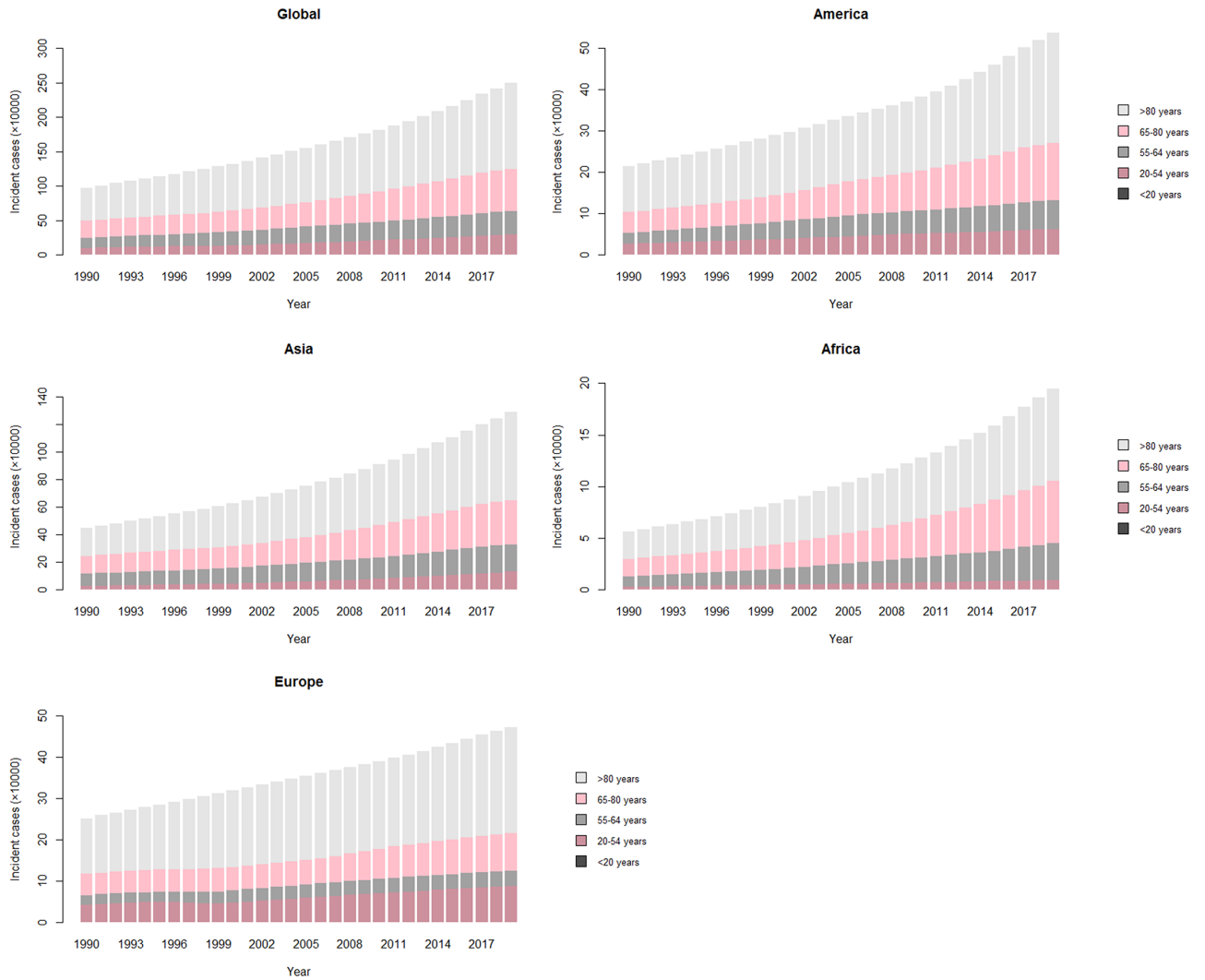

FIGURE 2 | The number of type 2 diabetes–related CKD incident cases over 30 years. The vertical axis is the incident cases of type 2 diabetes–related CKD in four world regions (America, Asia, Africa, and Europe). The horizontal axis represents 30 years (1990–2019). Each column is the total number of incident cases among five age-groups (>80 years, 65–80 years, 55–64 years, 20–54 years, and <20 years) that year. CKD, chronic kidney disease.

Table 3). As for CKD-T2DM, it increased most rapidly in Central Asia (EAPC = 1.76, 95% CI: 1.58–1.93). YLDs rate of CKD-T2DM-related anemia increased only in low SDI quintile (EAPC = 0.50, 95% CI: 0.46–0.54), and it increased the fastest in High-income North America (EAPC = 1.20, 95% CI: 0.90–1.50, **Table S7**).

The prevalence and YLD rate of CKD-T2DM and CKD-T1DM was different among sex and age (**Figure 5**). The main onset age of CKD-T1DM-related anemia changed from 15–19 years in 1990 to 15–39 years for females. But that for males was stable, with two peaks at 15–19 and 55–59 years. The YLD rate of CKD-T1DM-related anemia was higher in females aged 15–24 years and in males aged 10–14 years. As for CKD-T2DM-related anemia, the prevalence and YLD rate increased with age.

DISCUSSION

This study investigated the global, regional, and national disease burden of CKD-DM. Globally in 2019, there were 2.6 million incident cases, 135 million patients, 0.5 million deaths, and 13 million DALYs of CKD-DM, with a large increment as the global population grew. Further analysis showed that CKD-T2DM accounted for 95.32, 96.27, 83.20, and 75.38% of total CKD-DM incident cases, patients, deaths, and DALYs, respectively, reflecting the key role of type 2 diabetes in CKD development (20). All ASRs of CKD-T2DM increased from 1990 to 2019. All measured rates of CKD-T2DM increased with age, mirroring the cumulative risk effects of age. From the age of 50, all rates were higher in males than females. Interestingly, ASIR and ASPR of

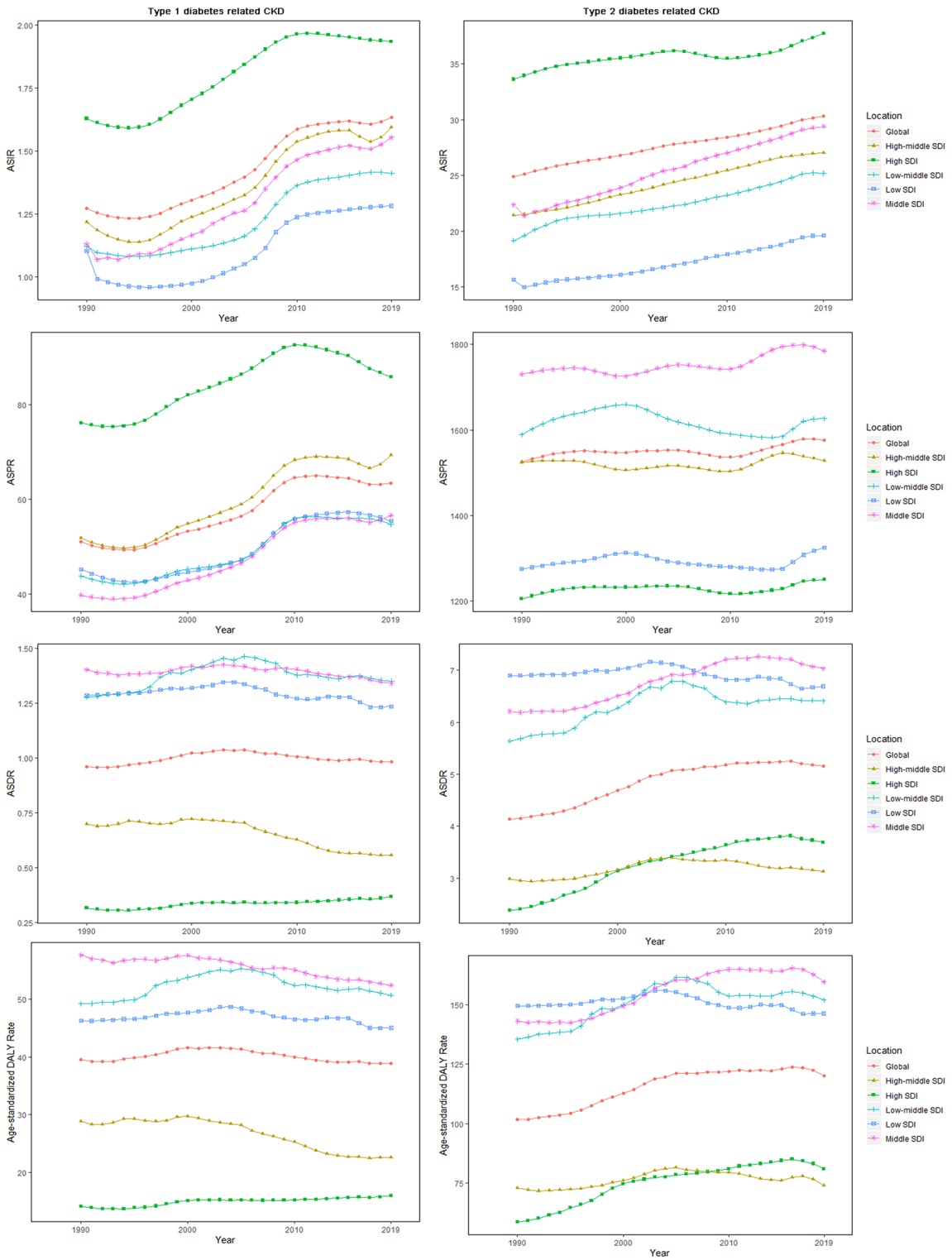

FIGURE 3 | The age-standardized rates for CKD-DM among SDI quintiles over 30 years. The vertical axis is the age-standardized incidence, prevalence, death, and DALY rate (per 100,000 person-years), and the horizontal axis is the 30 years (1990–2019). Each point represents the age-standardized incidence, prevalence, death, and DALY rate (per 100,000 person-years) that year. Each color and shape represents an SDI quintile (Global, High SDI, High-middle SDI, Middle SDI, Low-middle SDI, and Low SDI). CKD-DM, type 1 diabetes–related chronic kidney disease; DALY, disability adjusted life-year; ASIR, age-standardized incidence rate; ASPR, age-standardized prevalence rate; ASDR, age-standardized death rate; SDI, socio-demographic index.

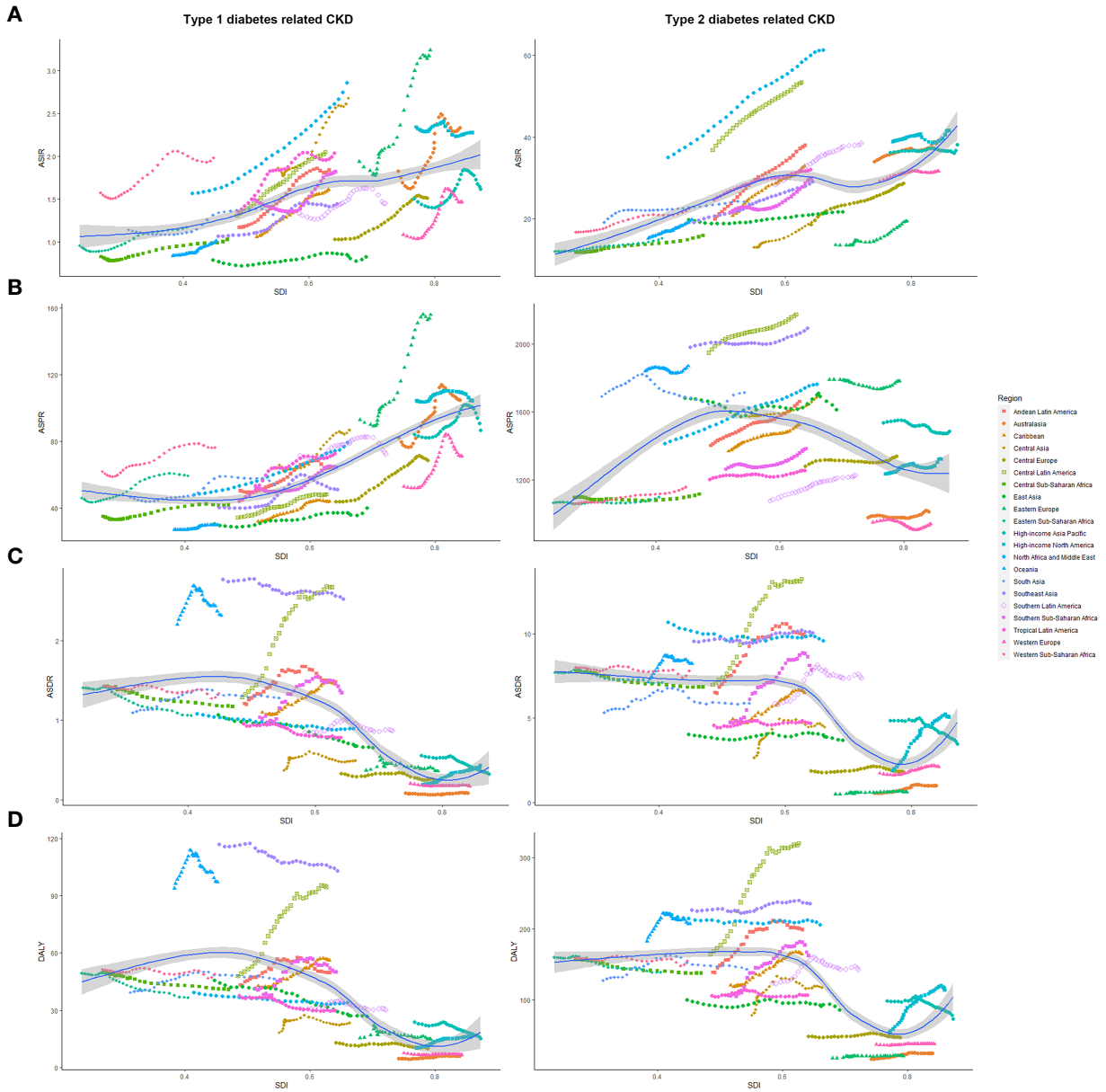

FIGURE 4 | The age-standardized rates of CKD-DM among 21 regions based on SDI in 2019. The vertical axis is the age-standardized incidence, prevalence, death, and DALY rate (per 100,000 person-years), and the horizontal axis is the SDI value in 2019. Each combination of colors and shapes represents a region, 21 in total. Each point represents the age-standardized incidence, prevalence, death, and DALY rate (per 100,000 person-years) that year in this region. Each combination of the same color and shape, from front to back, is the data for each year from 1990 to 2019. **(A)** ASIR (per 100,000 population); **(B)** ASDR (per 100,000 population); **(C)** ASPR (per 100,000 population); **(D)** Age-standardized DALY rate (per 100,000 population). ASIR, age-standardized incidence rate; ASPR, age-standardized prevalence rate; ASDR, age-standardized death rate; CKD-DM, diabetes-related chronic kidney disease; DALY, disability adjusted life-year; SDI, socio-demographic index.

CKD-T1DM increased globally, whereas ASDR and age-standardized DALY rate decreased for women but increased for men. As for the sex difference, sex hormones had a vital role in the development of diabetes and renal complications (21). In a previous study, racial differences were observed between women and men in diabetes, and the relationship between life course and diabetes was peculiar to women (22).

In High-income North America, Eastern Europe, North Africa, and Middle East, ASIR of CKD-T1DM kept higher than other regions. One study reported that the incidence of ESRD in diabetic patients was 10 times higher than non-diabetic patients. In Australia, one of the high-income countries, diabetes had become the leading cause of ESRD over the past 20 years (23). The mortality in ESRD patients was 18.3 times higher than

TABLE 3 | The prevalent cases and ASPR of impairment caused by diabetes mellitus–related chronic kidney disease.

Location	Impairment	Sex	Diabetes mellitus type 1–related chronic kidney disease					Diabetes mellitus type 2–related chronic kidney disease				
			Patient cases (No. ×1000) (95% UI)		ASPR (95% UI)		EAPC (95% CI)	Patient cases (No. ×1000) (95% UI)		ASPR (95% UI)		EAPC (95% CI)
			1990	2019	1990	2019		1990	2019	1990	2019	
Global	Anemia	Both	359.39(408.79-314.74)	655.24(741.99-577.15)	6.58(5.81-7.43)	8.48(7.46-9.57)	1.3(1.15-1.46)	3455.45(3817.46-3116.84)	8243.06(9070.84-7466.37)	89.15(80.39-98.24)	101.69(92.25-111.69)	0.54(0.51-0.57)
		Female	188.46(214.93-164.87)	358.02(410.74-311.18)	6.86(6.03-7.82)	9.29(8.08-10.6)	1.44(1.29-1.58)	1753.15(1936.67-1580.67)	4003.13(4419.19-3603.71)	81.47(73.5-89.79)	92.01(82.98-101.48)	0.54(0.51-0.58)
		Male	170.92(195.9-149.12)	297.22(337.86-261.36)	6.33(5.56-7.23)	7.72(6.77-8.77)	1.16(1-1.32)	1702.3(1893.46-1520.71)	4239.93(4682.53-3824.64)	101.94(91.43-112.61)	115.61(104.46-127.43)	0.48(0.45-0.5)
	Mild anemia	Both	180.23(202.72-159.05)	371.01(422.29-327.67)	3.39(3.01-3.83)	4.71(4.16-5.34)	1.63(1.46-1.8)	1934.53(2134.57-1741.24)	5126.35(5645.34-4643.76)	49.94(45.05-55.02)	63.21(57.37-69.6)	0.93(0.89-0.98)
		Female	90.03(102.7-78.83)	189.47(217.77-164.64)	3.29(2.89-3.74)	4.87(4.24-5.6)	1.75(1.6-1.89)	802.38(883.91-722.93)	2037.82(2249.03-1835.13)	37.23(33.59-41.05)	46.88(42.23-51.66)	0.98(0.93-1.04)
		Male	90.2(102.9-79.29)	181.54(206.66-159.04)	3.51(3.08-3.98)	4.6(4.04-5.22)	1.52(1.32-1.71)	1132.15(1257.05-1011.71)	3088.53(3415.83-2793.3)	67.56(60.59-74.75)	83.92(76.1-92.78)	0.82(0.79-0.85)
	Moderate anemia	Both	162.83(187-140.85)	263.86(301.92-230.18)	2.9(2.53-3.31)	3.5(3.04-4.01)	1(0.87-1.14)	1356.68(1501.94-1224.79)	2844.45(3148.81-2563.94)	34.97(31.56-38.55)	35.12(31.7-38.81)	0.07(0.04-0.1)
		Female	89.88(103.44-77.85)	157.39(181.2-136.39)	3.26(2.84-3.74)	4.12(3.58-4.74)	1.2(1.05-1.35)	854.03(944.94-770.7)	1804.56(2004.47-1622.38)	39.75(35.91-43.92)	41.45(37.28-45.99)	0.22(0.19-0.25)
		Male	72.95(85.55-62.29)	106.47(123.43-91.78)	2.55(2.2-2.96)	2.87(2.47-3.34)	0.73(0.61-0.86)	502.65(558.24-447.13)	1039.89(1150.01-934.35)	30.25(27.06-33.53)	28.61(25.83-31.54)	-0.18(-0.22–0.13)
	Severe anemia	Both	16.33(18.98-13.93)	20.36(23.64-17.62)	0.29(0.25-0.34)	0.27(0.23-0.32)	0.17(0.03-0.31)	164.24(182.67-147.46)	272.27(304.6-244.69)	4.24(3.81-4.71)	3.36(3.02-3.75)	-0.83(-0.92–0.75)
		Female	8.56(10.09-7.25)	11.15(12.97-9.57)	0.31(0.27-0.36)	0.29(0.25-0.34)	0.24(0.07-0.41)	96.74(108.01-86.41)	160.75(180.23-144.72)	4.49(4.02-5.02)	3.68(3.32-4.13)	-0.66(-0.72–0.59)
		Male	7.77(9.17-6.51)	9.21(11.11-7.74)	0.27(0.23-0.32)	0.25(0.21-0.3)	0.08(-0.05-0.2)	67.5(75.48-59.96)	111.51(125.42-99.63)	4.13(3.69-4.62)	3.08(2.76-3.43)	-1.13(-1.24–1.02)
Socio-demographic index												
High SDI	Anemia	Both	52.24(61.6-44.76)	72.01(82.87-62.09)	6.07(5.19-7.2)	6.29(5.41-7.31)	0.49(0.37-0.62)	851.24(949.21-766.96)	1652.9(1853.2-1484.69)	79.86(72.07-88.83)	83.3(74.85-93.14)	0.2(0.14-0.26)
		Female	31.56(37.76-26.76)	40.54(47.37-34.79)	7.25(6.11-8.71)	7.62(6.47-9.06)	0.44(0.29-0.58)	431.97(483.64-385.73)	741.18(837.2-650.87)	69.28(61.88-77.22)	69.47(61.05-78.43)	0.11(0.03-0.19)
		Male	20.68(24.32-17.61)	31.47(37.24-26.62)	5.05(4.27-5.96)	5.16(4.34-6.11)	0.61(0.39-0.84)	419.28(472.27-372.61)	911.72(1039.8-800.91)	99.43(89.08-111.35)	102.84(90.54-116.58)	0.11(0.07-0.15)
	Mild anemia	Both	39.08(45.95-33.45)	56.71(65.28-48.98)	4.49(3.85-5.31)	4.87(4.19-5.66)	0.66(0.53-0.78)	627.3(699.34-562.94)	1274.6(1432.5-1144.21)	58.8(53.02-65.44)	64.66(58.21-72.31)	0.36(0.32-0.4)
		Female	21.94(26.32-18.51)	29.27(34.3-25.13)	5.04(4.23-6.06)	5.53(4.68-6.58)	0.57(0.41-0.73)	273.95(306.07-243.12)	495.43(556.02-438.86)	44.14(39.4-49.37)	47.11(41.91-53.1)	0.31(0.25-0.36)
		Male	17.14(20.15-14.65)	27.44(32.62-23.08)	4.08(3.48-4.81)	4.36(3.68-5.14)	0.78(0.57-1)	353.36(398.56-313.89)	779.16(887.15-686.78)	83.14(74.16-93.35)	88.04(77.67-99.83)	0.17(0.14-0.21)
	Moderate anemia	Both	12.72(15.3-10.72)	14.83(17.24-12.76)	1.53(1.28-1.84)	1.38(1.18-1.62)	-0.01(-0.13-0.11)	214.27(240.57-190.65)	364.08(414.81-317.87)	20.15(17.99-22.52)	17.95(15.75-20.43)	-0.28(-0.39–0.17)
		Female	9.31(11.27-7.82)	10.94(12.92-9.31)	2.14(1.79-2.61)	2.03(1.71-2.38)	0.11(-0.01-0.23)	151.97(172.13-132.96)	238.07(276.2-203.54)	24.18(21.36-27.29)	21.68(18.53-25)	-0.24(-0.35–0.13)
		Male	3.41(4.29-2.75)	3.89(4.73-3.23)	0.93(0.73-1.18)	0.78(0.61-1)	-0.19(-0.46-0.08)	62.3(72-53.59)	126(152.96-105.08)	15.37(13.32-17.56)	14.07(11.77-16.98)	-0.21(-0.32–0.1)
	Severe anemia	Both	0.44(0.56-0.36)	0.47(0.56-0.4)	0.05(0.04-0.07)	0.04(0.03-0.05)	-0.4(-0.56–0.24)	9.67(10.93-8.5)	14.23(16.64-12.15)	0.91(0.81-1.02)	0.69(0.59-0.8)	-0.79(-0.97–0.6)
		Female	0.31(0.39-0.25)	0.33(0.4-0.27)	0.07(0.06-0.09)	0.06(0.05-0.07)	-0.2(-0.33–0.07)	6.05(6.99-5.19)	7.67(9.39-6.17)	0.95(0.82-1.09)	0.68(0.56-0.83)	-0.92(-1.11–0.73)
		Male	0.13(0.17-0.1)	0.14(0.17-0.12)	0.03(0.03-0.04)	0.02(0.02-0.03)	-0.77(-1.07–0.47)	3.62(4.21-3.09)	6.56(8.06-5.38)	0.91(0.78-1.06)	0.73(0.6-0.9)	-0.64(-0.8–0.48)
High-middle SDI	Anemia	Both	67.76(79.87-57.64)	104.99(124.02-87.91)	5.83(4.96-6.88)	7.07(5.93-8.49)	1.1(0.94-1.25)	785.88(873.46-707.68)	1610.42(1787.07-1451.96)	74.87(67.54-82.85)	79.26(71.58-87.83)	0.34(0.3-0.38)
		Female	35.62(42.52-29.95)	59.93(73.15-49.26)	6(5.06-7.16)	8.33(6.8-10.33)	1.58(1.43-1.74)	405.53(451.8-363.44)	800.41(888.37-719.69)	67.38(60.53-74.78)	71.98(65.04-79.97)	0.43(0.38-0.49)
		Male	32.14(38.39-27.09)	45.06(53.98-37.48)	5.74(4.86-6.83)	5.98(5.01-7.2)	0.56(0.4-0.73)	380.35(424.82-340.71)	810.01(900.84-727.65)	89.34(80.12-98.78)	91.61(82.52-101.44)	0.18(0.15-0.21)
	Mild anemia	Both	40.41(46.95-34.45)	72.14(85.25-60.58)	3.45(2.95-4.02)	4.71(3.94-5.62)	1.6(1.42-1.78)	475.87(529.72-426.95)	1114.64(1234.89-1004.58)	45.03(40.57-49.95)	54.8(49.5-60.57)	0.89(0.83-0.95)
		Female	19.47(22.88-16.45)	37.2(45.26-30.63)	3.25(2.74-3.82)	5.17(4.18-6.37)	2.15(1.97-2.33)	200.98(224.64-179.72)	461.96(513.39-415.6)	33.2(29.79-37.07)	41.65(37.52-46.24)	1.11(1.02-1.2)
		Male	20.94(24.6-17.8)	34.94(41.35-29.16)	3.71(3.17-4.35)	4.37(3.68-5.19)	1.09(0.89-1.29)	274.89(306.41-245.12)	652.68(731.46-584.46)	63.73(57-70.83)	73.45(65.87-81.59)	0.63(0.59-0.66)
	Moderate anemia	Both	25.74(31.24-21.31)	31.36(37.81-26.03)	2.25(1.86-2.72)	2.26(1.84-2.78)	0.32(0.21-0.42)	287.09(318.07-258.16)	468.73(520.85-422.78)	27.62(25-30.52)	23.12(20.9-25.6)	-0.58(-0.62–0.53)
		Female	15.2(18.59-12.53)	21.75(26.85-17.79)	2.59(2.12-3.17)	3.03(2.45-3.77)	0.88(0.76-1.01)	190.14(211.84-170.5)	321.73(360.13-288.54)	31.76(28.55-35.31)	28.84(25.91-32.23)	-0.25(-0.3–0.2)
		Male	10.54(13.04-8.46)	9.61(11.99-7.76)	1.91(1.53-2.37)	1.53(1.2-1.97)	-0.53(-0.62–0.44)	96.95(108.64-85.83)	147(167.22-129.97)	23.48(20.94-26.18)	16.96(15.07-19.19)	-1.17(-1.26–1.07)
	Severe anemia	Both	1.62(2.04-1.31)	1.49(1.86-1.21)	0.14(0.11-0.18)	0.1(0.08-0.13)	-0.88(-0.97–0.79)	22.92(25.95-20.31)	27.04(30.62-23.94)	2.23(1.99-2.51)	1.34(1.18-1.51)	-1.85(-1.95–1.75)
		Female	0.96(1.22-0.76)	0.98(1.24-0.77)	0.16(0.13-0.21)	0.13(0.1-0.17)	-0.51(-0.61–0.41)	14.41(16.47-12.58)	16.72(19.15-14.55)	2.42(2.11-2.75)	1.5(1.31-1.72)	-1.71(-1.8–1.61)
		Male	0.66(0.85-0.53)	0.52(0.68-0.41)	0.12(0.1-0.15)	0.08(0.06-0.1)	-1.39(-1.5–1.28)	8.51(9.83-7.41)	10.33(11.91-8.96)	2.12(1.85-2.42)	1.2(1.04-1.39)	-2.07(-2.19–1.96)
Low SDI	Anemia	Both	41.48(50.15-34.05)	121.24(146.13-99.29)	7.72(6.46-9.16)	10.34(8.65-12.22)	1.54(1.33-1.75)	180.83(200.62-161.61)	506.59(560.59-452.61)	75.84(67.96-84.34)	99.27(88.72-110.21)	1.01(0.97-1.05)
		Female	20.24(24.42-16.41)	63.21(76.7-51.03)	7.84(6.44-9.36)	10.95(8.97-13.11)	1.64(1.42-1.86)	94.43(104.96-84.39)	261.85(289.29-235.6)	75.78(67.75-84.07)	95.88(85.94-107.04)	0.86(0.83-0.9)
		Male	21.24(26.14-17.2)	58.03(70.97-47.35)	7.54(6.33-8.99)	9.67(8.11-11.48)	1.44(1.23-1.65)	86.4(96.98-76.08)	244.74(273-217.45)	75.79(67.19-84.96)	103.29(92.17-114.82)	1.18(1.14-1.23)
	Mild anemia	Both	13.61(16.09-11.3)	48.02(56.96-39.68)	2.86(2.4-3.37)	4.45(3.72-5.24)	2.2(1.94-2.45)	66.01(73.69-59.15)	214.2(237.87-192.17)	26.44(23.62-29.6)	40.13(36.07-44.57)	1.58(1.49-1.66)
		Female	6.24(7.5-5.09)	24.04(29.12-19.34)	2.54(2.09-3.01)	4.27(3.48-5.13)	2.34(2.1-2.58)	26.72(29.87-23.92)	84.87(94.07-76.01)	20.08(17.98-22.4)	29.23(26.12-32.45)	1.38(1.3-1.46)
		Male	7.37(8.76-6.13)	23.98(28.8-19.83)	3.15(2.65-3.75)	4.62(3.88-5.46)	2.09(1.82-2.36)	39.29(44.41-34.65)	129.33(144.5-115.04)	32.57(28.78-36.72)	51.7(46.01-57.85)	1.77(1.68-1.86)
	Moderate anemia	Both	24.03(29.49-19.53)	65.78(80.58-52.96)	4.21(3.51-5.02)	5.29(4.4-6.33)	1.25(1.07-1.44)	93.81(104.14-83.73)	250.83(279.06-223.82)	39.95(35.63-44.64)	50.3(44.88-56.04)	0.89(0.86-0.92)
		Female	12.13(14.76-9.79)	35.41(43.16-28.39)	4.6(3.8-5.51)	6.04(4.93-7.24)	1.41(1.2-1.62)	55.9(62.03-49.97)	152.89(170.29-137.03)	45.6(40.71-50.71)	57.16(51.08-63.85)	0.84(0.81-0.87)
		Male	11.9(14.98-9.32)	30.37(38.12-24.02)	3.78(3.09-4.59)	4.5(3.69-5.48)	1.05(0.88-1.22)	37.91(42.81-33.28)	97.93(109.86-85.98)	34.34(30.43-38.58)	43.35(38.24-48.56)	0.94(0.89-0.98)
	Severe anemia	Both	3.84(4.79-3.03)	7.44(9.22-5.99)	0.66(0.54-0.79)	0.6(0.49-0.72)	0.11(-0.05-0.27)	21.01(23.69-18.46)	41.56(46.98-36.62)	9.45(8.34-10.69)	8.83(7.81-10)	-0.26(-0.38–0.14)
		Female	1.87(2.34-1.47)	3.76(4.7-3.04)	0.7(0.57-0.86)	0.64(0.52-0.78)	0.17(-0.02-0.36)	11.81(13.33-10.33)	24.08(27.54-21.22)	10.1(8.84-11.4)	9.49(8.33-10.82)	-0.2(-0.28–0.12)
		Male	1.97(2.51-1.51)	3.68(4.66-2.83)	0.61(0.49-0.75)	0.54(0.44-0.67)	0.05(-0.09-0.19)	9.2(10.72-7.89)	17.48(20.2-14.95)	8.89(7.7-10.21)	8.25(7.14-9.48)	-0.31(-0.47–0.15)
Low-middle SDI	Anemia	Both	85.66(106.02-69.86)	170.49(206.95-140.93)	7.27(6.07-8.76)	9.39(7.82-11.27)	1.35(1.18-1.52)	589.68(656.07-526.87)	1612.87(1788.61-1449.51)	98.06(87.77-108.82)	121.02(108.75-134.17)	0.64(0.56-0.72)
		Female	41.29(52.23-33.33)	88.1(108.29-71.78)	7.09(5.8-8.75)	9.54(7.83-11.63)	1.4(1.25-1.55)	283.62(314.22-254.9)	773.3(860.3-691.54)	90.9(81.46-101.31)	109.12(97.86-121.5)	0.53(0.47-0.6)
		Male	44.37(54.62-35.76)	82.38(100.12-67.7)	7.41(6.19-8.94)	9.23(7.67-11.06)	1.33(1.14-1.52)	306.06(342.55-271.96)	839.57(928.54-754.58)	105.57(94.16-117.75)	135.39(121.77-150.12)	0.78(0.69-0.87)
	Mild anemia	Both	31.45(37.98-26.13)	80.76(96.5-67.27)	2.82(2.38-3.34)	4.43(3.73-5.26)	2.08(1.9-2.27)	223.92(249.74-200.37)	744.36(823.74-668.95)	35.81(32.03-39.93)	54.88(49.48-60.59)	1.48(1.43-1.53)
		Female	14.15(17.56-11.55)	38.69(47.55-31.62)	2.44(2.02-2.97)	4.14(3.4-5.05)	2.15(2.01-2.3)	82.16(91.6-73.39)	273.27(304.01-243.73)	25.1(22.43-27.94)	38.01(33.88-42.4)	1.45(1.42-1.47)
		Male	17.3(20.81-14.33)	42.07(49.99-35.21)	3.18(2.69-3.81)	4.74(3.99-5.61)	2.07(1.84-2.3)	141.75(159.65-125.73)	471.09(522.64-421.42)	46.49(41.38-52.08)	73.95(66.13-82.2)	1.61(1.55-1.67)
	Moderate anemia	Both	47.78(59.73-38.38)	82.83(102.07-67.53)	3.91(3.22-4.76)	4.57(3.76-5.57)	0.97(0.82-1.12)	305.58(340.62-272.43)	763(851.64-682.72)	51.52(46.02-57.39)	57.9(51.92-64.4)	0.29(0.19-0.39)
		Female	23.91(30.25-19.14)	45.74(56.47-37.07)	4.08(3.32-5.06)	4.99(4.08-6.11)	1.11(0.95-1.26)	168.38(187.15-150.77)	439.46(491.64-391.48)	54.62(48.99-60.88)	62.32(55.67-69.85)	0.34(0.26-0.42)
		Male	23.87(30.15-18.76)	37.1(46.67-29.69)	3.72(3.03-4.57)	4.13(3.34-5.12)	0.82(0.67-0.97)	137.2(154.2-121.02)	323.55(361.82-288.66)	48.69(43.21-54.76)	53.71(48.01-59.73)	0.24(0.11-0.37)
	Severe anemia	Both	6.43(8.35-5.06)	6.89(8.79-5.57)	0.54(0.44-0.68)	0.39(0.32-0.49)	-0.61(-0.79–0.42)	60.19(67.41-53.54)	105.52(118.78-94.13)	10.73(9.57-12.02)	8.24(7.37-9.24)	-1.11(-1.3–0.93)
		Female	3.22(4.29-2.48)	3.68(4.71-2.96)	0.57(0.45-0.74)	0.41(0.33-0.52)	-0.62(-0.82–0.42)	33.09(37.1-29.54)	60.58(68.19-54.07)	11.19(9.99-12.54)	8.8(7.87-9.89)	-0.98(-1.14–0.81)
		Male	3.2(4.2-2.48)	3.21(4.27-2.52)	0.51(0.42-0.64)	0.37(0.29-0.48)	-0.59(-0.77–0.41)	27.1(30.75-23.57)	44.94(51.19-39.45)	10.39(9.11-11.75)	7.73(6.83-8.75)	-1.29(-1.5–1.07)
Middle SDI	Anemia	Both	112.05(133.07-94.68)	186.11(221.96-159.74)	6.1(5.23-7.11)	7.83(6.68-9.32)	1.29(1.13-1.45)	1045.71(1168.11-928.1)	2854.16(3171.57-2557.21)	103.37(91.89-114.95)	118.4(106.73-131.35)	0.6(0.55-0.66)
		Female	59.66(71.52-50.2)	106.02(129.11-90.14)	6.38(5.41-7.58)	8.91(7.56-10.88)	1.57(1.4-1.74)	536.54(597.26-479.14)	1423.44(1585.78-1276.81)	98.16(87.22-108.99)	111.03(99.92-123.15)	0.61(0.53-0.69)
		Male	52.4(62.64-44.35)	80.1(93.84-69.01)	5.82(4.96-6.79)	6.77(5.8-7.96)	0.97(0.82-1.13)	509.17(573.16-447.99)	1430.73(1588.35-1279.98)	111.07(97.74-123.87)	128.71(115.71-142.57)	0.59(0.56-0.63)
	Mild anemia	Both	55.58(65.71-47.25)	113.15(134.33-97.1)	3.07(2.64-3.61)	4.65(4-5.56)	1.9(1.73-2.07)	540.13(604.03-477.72)	1774.42(1970.45-1592.8)	51.68(45.73-57.78)	72.82(65.65-80.75)	1.35(1.27-1.44)
		Female	28.18(34.07-23.76)	60.15(73.89-50.45)	2.97(2.53-3.54)	5.03(4.23-6.25)	2.21(2.05-2.37)	218.06(245.02-193.9)	720.7(803.21-644.88)	38.23(33.89-42.81)	55.89(50.28-62.12)	1.57(1.44-1.7)
		Male	27.41(32.25-23.36)	53(62.3-45.7)	3.17(2.74-3.69)	4.31(3.72-5.03)	1.61(1.42-1.8)	322.07(363.55-282.85)	1053.72(1175.68-942.72)	67.58(59.2-76.04)	93.12(83.9-103.53)	1.22(1.16-1.27)
	Moderate anemia	Both	52.48(62.77-44.06)	68.91(82.14-58.34)	2.81(2.39-3.32)	3(2.53-3.61)	0.62(0.47-0.76)	455.18(509.84-405.02)	995.93(1108.27-889.76)	46.24(41.23-51.52)	41.96(37.69-46.59)	-0.23(-0.27–0.19)
		Female	29.28(34.94-24.52)	43.46(52.48-36.85)	3.16(2.68-3.75)	3.68(3.09-4.47)	0.96(0.78-1.14)	287.13(320.03-255.76)	651.11(730.3-582.52)	53.78(47.9-59.98)	51.03(45.82-57.1)	-0.05(-0.11–0.02)
		Male	23.19(27.96-19.27)	25.44(30.85-21.28)	2.45(2.05-2.91)	2.31(1.91-2.83)	0.1(-0.01-0.21)	168.05(189.96-148.34)	344.83(385.88-305.9)	38.73(34.4-43.37)	32.44(28.94-36.2)	-0.56(-0.63–0.49)
	Severe anemia	Both	3.99(4.74-3.31)	4.06(4.86-3.44)	0.22(0.19-0.26)	0.18(0.15-0.21)	-0.21(-0.4–0.01)	50.4(56.79-44.7)	83.81(94.71-74.55)	5.45(4.84-6.14)	3.62(3.22-4.08)	-1.33(-1.4–1.27)
		Female	2.19(2.64-1.83)	2.41(2.87-2.01)	0.24(0.21-0.29)	0.2(0.17-0.24)	-0.05(-0.28-0.19)	31.35(35.45-27.6)	51.63(58.59-45.63)	6.16(5.41-7.03)	4.11(3.64-4.67)	-1.26(-1.31–1.21)
		Male	1.8(2.19-1.47)	1.65(2.03-1.35)	0.2(0.16-0.23)	0.15(0.12-0.18)	-0.44(-0.59–0.28)	19.05(21.76-16.64)	32.18(36.57-28.24)	4.76(4.2-5.42)	3.15(2.78-3.56)	-1.45(-1.55–1.35)
Region												
Andean Latin America	Anemia	Both	3.08(5.24-1.73)	6.99(11.53-4.16)	7.6(4.72-12.1)	10.74(6.48-17.5)	1.56(1.4-1.73)	14.18(15.93-12.59)	56.7(63.99-49.88)	67.56(59.85-75.89)	102.22(89.82-115.7)	1.38(1.26-1.49)
	Mild anemia	Both	1.4(2.35-0.83)	4.38(7.1-2.62)	3.62(2.34-5.6)	6.71(4.06-10.76)	2.62(2.4-2.83)	7.38(8.34-6.5)	36.91(41.8-32.65)	34.94(30.82-39.48)	66.33(58.59-75.15)	2.15(1.97-2.34)
	Moderate anemia	Both	1.55(2.69-0.85)	2.5(4.2-1.42)	3.68(2.17-6.04)	3.86(2.21-6.43)	0.37(0.24-0.51)	6.11(6.93-5.32)	18.31(20.92-15.8)	29.18(25.49-32.97)	33.18(28.48-37.9)	0.38(0.32-0.44)
	Severe anemia	Both	0.12(0.22-0.07)	0.11(0.18-0.07)	0.31(0.18-0.5)	0.17(0.11-0.27)	-1.67(-1.91–1.43)	0.7(0.82-0.59)	1.48(1.73-1.25)	3.44(2.92-4.04)	2.72(2.3-3.16)	-0.79(-0.88–0.69)
Australasia	Anemia	Both	1.43(2.17-0.98)	2.56(3.83-1.77)	6.68(4.5-10.31)	7.96(5.17-12.43)	0.99(0.75-1.24)	15.77(18.41-13.23)	32.13(39.53-25.81)	66.61(56.58-77.13)	61.01(49.15-74.53)	-0.37(-0.46–0.29)
	Mild anemia	Both	1.08(1.6-0.74)	2.03(3.03-1.39)	5.02(3.4-7.62)	6.24(4.03-9.68)	1.15(0.9-1.41)	11.76(13.83-9.82)	25.44(31-20.4)	49.33(41.76-57.41)	48.44(38.94-59)	-0.14(-0.21–0.07)

(Continued)

TABLE 3 | Continued

Location	Impairment	Sex	Diabetes mellitus type 1–related chronic kidney disease					Diabetes mellitus type 2–related chronic kidney disease				
			Patient cases (No. ×1000) (95% UI)		ASPR (95% UI)		EAPC (95% CI)	Patient cases (No. ×1000) (95% UI)		ASPR (95% UI)		EAPC (95% CI)
			1990	2019	1990	2019		1990	2019	1990	2019	
	Moderate anemia	Both	0.34(0.53-0.22)	0.51(0.79-0.34)	1.61(1.03-2.59)	1.68(1.02-2.71)	0.46(0.27-0.66)	3.84(4.84-2.96)	6.43(8.33-4.79)	16.53(13.01-20.67)	12.08(9.11-15.57)	-1.14(-1.31–0.98)
	Severe anemia	Both	0.01(0.02-0.01)	0.01(0.02-0.01)	0.05(0.03-0.07)	0.04(0.03-0.07)	0.12(-0.09-0.32)	0.17(0.23-0.13)	0.27(0.37-0.19)	0.74(0.56-0.97)	0.49(0.36-0.68)	-1.44(-1.65–1.22)
Caribbean	Anemia	Both	2.47(3.76-1.65)	4.71(6.98-3.31)	6.66(4.63-9.77)	10.09(6.96-15.15)	1.62(1.52-1.72)	32.35(36.38-28.61)	91.04(102.18-80.41)	123(108.99-138.76)	176.23(155.77-197.55)	1.15(1.06-1.25)
	Mild anemia	Both	1.29(1.91-0.89)	2.69(3.75-1.98)	3.55(2.57-5.06)	5.63(4.06-7.95)	1.84(1.69-1.99)	20.82(23.54-18.36)	63.5(71.42-56.12)	79.09(69.83-89.63)	122.92(108.81-138.42)	1.46(1.34-1.57)
	Moderate anemia	Both	1.12(1.73-0.72)	1.93(3.18-1.23)	2.95(1.94-4.46)	4.27(2.66-7.17)	1.38(1.33-1.44)	10.88(12.39-9.58)	26.24(29.8-22.95)	41.46(36.55-47.15)	50.78(44.48-57.61)	0.57(0.51-0.63)
	Severe anemia	Both	0.06(0.1-0.04)	0.09(0.16-0.05)	0.16(0.11-0.27)	0.2(0.11-0.37)	0.61(0.51-0.71)	0.65(0.75-0.56)	1.31(1.53-1.11)	2.45(2.12-2.83)	2.53(2.16-2.94)	-0.03(-0.1-0.03)
Central Asia	Anemia	Both	7.77(11.72-5.2)	14.32(21.26-10.05)	11(7.66-16.02)	14.93(10.51-22.18)	1.42(1.13-1.72)	38.91(44.34-34.29)	93.11(105.42-82.4)	78.5(69.28-88.83)	123.17(109.52-138.69)	1.76(1.58-1.93)
	Mild anemia	Both	3.6(5.2-2.47)	7.88(11.6-5.6)	5.22(3.68-7.27)	8.25(5.89-12.17)	2.15(1.82-2.48)	18.31(20.81-16.04)	50.56(57.42-44.81)	37.04(32.6-42.03)	67.28(59.75-75.71)	2.34(2.11-2.57)
	Moderate anemia	Both	3.9(6.17-2.49)	6.09(9.31-4.12)	5.39(3.62-8.25)	6.31(4.3-9.63)	0.74(0.47-1)	18.89(21.76-16.57)	39.75(45.21-34.84)	37.98(33.45-43)	52.2(45.99-58.8)	1.25(1.13-1.37)
	Severe anemia	Both	0.27(0.41-0.18)	0.35(0.52-0.25)	0.39(0.28-0.57)	0.36(0.26-0.54)	-0.03(-0.2-0.14)	1.71(2-1.47)	2.8(3.29-2.38)	3.48(2.99-4.02)	3.69(3.17-4.28)	0.19(0.16-0.22)
Central Europe	Anemia	Both	6.66(8.51-5.42)	8.42(10.35-6.77)	5.52(4.41-7.16)	7.56(5.98-9.63)	1.55(1.4-1.7)	59.62(67.45-52.15)	94.52(107.77-82.98)	41.1(36.2-46.18)	48.81(42.97-55.64)	0.52(0.48-0.56)
	Mild anemia	Both	4.37(5.51-3.56)	6.27(7.68-5.05)	3.56(2.86-4.57)	5.51(4.37-7)	2.03(1.84-2.21)	42.3(47.57-37.05)	72.54(82.32-63.88)	29.14(25.73-32.67)	37.22(32.9-42.34)	0.77(0.72-0.82)
	Moderate anemia	Both	2.18(2.83-1.73)	2.08(2.59-1.65)	1.87(1.46-2.47)	1.98(1.55-2.57)	0.52(0.42-0.62)	16.27(18.89-13.94)	20.91(24.31-17.9)	11.22(9.72-12.97)	11.05(9.45-12.83)	-0.13(-0.18–0.08)
	Severe anemia	Both	0.1(0.13-0.08)	0.08(0.1-0.06)	0.09(0.07-0.11)	0.07(0.05-0.09)	-0.44(-0.57–0.32)	1.06(1.27-0.89)	1.07(1.27-0.91)	0.73(0.62-0.87)	0.54(0.46-0.65)	-1.17(-1.25–1.08)
Central Latin America	Anemia	Both	10.34(14.3-7.81)	17.68(22.71-14.04)	5.48(4.23-7.29)	7.05(5.6-9.06)	0.96(0.89-1.04)	111.58(124.76-98.46)	368.87(408.67-333.47)	131.87(116.72-148.16)	158.16(143.14-175.29)	0.57(0.52-0.62)
	Mild anemia	Both	5.9(8.01-4.45)	11.72(15.06-9.38)	3.22(2.49-4.23)	4.63(3.7-5.95)	1.33(1.22-1.45)	68.2(76.28-60.17)	251.86(277.82-227.31)	78.85(69.85-88.57)	107.32(97.14-118.73)	0.96(0.87-1.05)
	Moderate anemia	Both	4.21(5.82-3.16)	5.71(7.47-4.48)	2.14(1.64-2.89)	2.33(1.82-3.05)	0.38(0.34-0.42)	39.77(44.55-35.01)	108.71(121.2-97.58)	48.35(42.67-54.31)	47.17(42.41-52.66)	-0.09(-0.11–0.07)
	Severe anemia	Both	0.23(0.3-0.17)	0.24(0.31-0.19)	0.12(0.09-0.16)	0.1(0.08-0.12)	-0.49(-0.62–0.37)	3.61(4.06-3.18)	8.3(9.24-7.43)	4.67(4.1-5.28)	3.66(3.27-4.08)	-0.72(-0.82–0.63)
Central Sub-Saharan Africa	Anemia	Both	3.35(6.1-1.79)	10.77(19.13-5.99)	5.62(3.43-9.48)	7.58(4.75-12.46)	1.35(1.14-1.56)	12.49(14.45-10.91)	38.48(44.01-33.75)	53.55(47.69-60.52)	73.31(65.06-82.41)	1.07(0.94-1.2)
	Mild anemia	Both	1.03(1.79-0.61)	4.41(7.51-2.63)	1.96(1.29-3.15)	3.42(2.25-5.4)	2.33(2.05-2.61)	4.86(5.64-4.19)	18.92(21.74-16.39)	20.16(17.48-23.23)	33.74(29.43-38.3)	1.86(1.63-2.09)
	Moderate anemia	Both	2.05(3.87-1.05)	5.89(10.83-3.05)	3.24(1.9-5.61)	3.86(2.29-6.71)	0.9(0.74-1.07)	6.77(7.91-5.87)	18(20.7-15.74)	29.58(26.05-33.73)	36.22(31.9-41.16)	0.66(0.59-0.73)
	Severe anemia	Both	0.28(0.56-0.13)	0.46(0.89-0.23)	0.42(0.22-0.79)	0.3(0.17-0.54)	-1.03(-1.23–0.84)	0.87(1.06-0.7)	1.56(1.9-1.29)	3.81(3.14-4.59)	3.35(2.76-4.08)	-0.56(-0.64–0.48)
East Asia	Anemia	Both	39.23(48.48-31.64)	32.54(39.02-26.89)	3.09(2.51-3.76)	2.14(1.76-2.59)	-0.92(-1.06–0.77)	686.62(772.27-609.6)	1257.38(1406.13-1119.81)	81.62(72.84-91.4)	62.83(56.2-70.14)	-0.54(-0.66–0.42)
	Mild anemia	Both	21.26(26.2-17.24)	23.96(28.69-19.82)	1.64(1.35-2)	1.52(1.26-1.83)	0.2(0.01-0.38)	374.3(425.13-330.18)	924.21(1043.45-821.69)	42.75(37.93-48.53)	45.77(41-51.33)	0.72(0.56-0.87)
	Moderate anemia	Both	16.85(20.97-13.48)	8.17(9.96-6.6)	1.36(1.09-1.68)	0.59(0.47-0.74)	-2.7(-2.8–2.6)	285.26(319.83-252.61)	316.67(364.56-276.5)	35.32(31.45-39.49)	16.21(14.2-18.66)	-2.51(-2.64–2.37)
	Severe anemia	Both	1.13(1.44-0.88)	0.41(0.52-0.32)	0.09(0.07-0.11)	0.03(0.02-0.04)	-3.92(-4.09–3.75)	27.07(31.75-22.98)	16.5(20.15-13.47)	3.55(3.03-4.16)	0.85(0.7-1.03)	-4.92(-5.2–4.63)
Eastern Europe	Anemia	Both	21.53(25.62-17.99)	35.15(42.17-29.21)	9.29(7.64-11.09)	15.29(12.49-18.54)	2.51(2.16-2.85)	176.09(200.15-154.05)	314.93(361.82-272.23)	64.08(56.4-72.34)	91.4(79.46-104.98)	1.34(1.26-1.42)
	Mild anemia	Both	14.24(16.8-11.93)	25.49(30.7-21.19)	6.14(5.08-7.29)	11.11(9.05-13.45)	2.96(2.58-3.36)	108.46(123.69-95.21)	212.8(245.15-183.55)	39.16(34.64-44.39)	62.1(54.06-71.24)	1.89(1.77-2.02)
	Moderate anemia	Both	6.92(8.4-5.69)	9.25(11.34-7.49)	2.99(2.42-3.68)	4.02(3.21-5)	1.54(1.29-1.8)	63.7(74.69-53.93)	97.04(115.11-80.94)	23.45(20.01-27.34)	27.84(23.29-32.79)	0.44(0.32-0.57)
	Severe anemia	Both	0.37(0.47-0.29)	0.41(0.54-0.31)	0.15(0.12-0.19)	0.17(0.12-0.22)	0.59(0.41-0.76)	3.93(4.91-3.15)	5.09(6.39-4.08)	1.46(1.19-1.78)	1.46(1.17-1.8)	-0.47(-0.72–0.23)
Eastern Sub-Saharan Africa	Anemia	Both	12.68(16.24-9.91)	37.49(48.39-29.62)	6.87(5.61-8.47)	9.36(7.62-11.6)	1.59(1.36-1.82)	42.53(47.74-37.7)	119.88(133.82-106.48)	56.4(49.93-63.44)	76.11(67.34-85.82)	1.1(1.01-1.2)
	Mild anemia	Both	4.43(5.47-3.58)	16.89(21.25-13.64)	2.79(2.31-3.37)	4.6(3.76-5.56)	2.47(2.17-2.76)	18.04(20.26-15.98)	59.93(67.05-52.88)	22.66(20-25.52)	36.21(31.94-40.66)	1.78(1.63-1.93)
	Moderate anemia	Both	7.22(9.46-5.52)	18.97(25.21-14.56)	3.6(2.88-4.52)	4.38(3.51-5.53)	1.08(0.89-1.27)	21.74(24.43-19.16)	54.94(61.96-48.59)	29.78(26.27-33.59)	36.51(32.21-41.45)	0.75(0.68-0.82)
	Severe anemia	Both	1.04(1.42-0.76)	1.62(2.17-1.23)	0.48(0.38-0.62)	0.38(0.3-0.48)	-0.72(-0.83–0.61)	2.75(3.11-2.43)	5.01(5.66-4.41)	3.96(3.51-4.48)	3.4(3-3.87)	-0.71(-0.77–0.64)
High-income Asia Pacific	Anemia	Both	12.02(15.93-9.13)	14.12(18.27-10.95)	6.5(4.96-8.57)	6.01(4.59-7.84)	0.33(0.11-0.55)	263.62(298.32-235.14)	590.06(686.75-515.42)	134.49(120.37-151.76)	118.84(104.32-137.74)	-0.57(-0.6–0.53)
	Mild anemia	Both	8.13(10.44-6.26)	10.9(14-8.49)	4.35(3.34-5.63)	4.55(3.51-5.9)	0.75(0.51-0.99)	178.86(201.34-157.14)	442.69(514.35-387.27)	90.29(79.79-101.29)	90.68(79.39-105.48)	-0.13(-0.19–0.07)
	Moderate anemia	Both	3.7(5.09-2.75)	3.12(4.08-2.32)	2.06(1.52-2.9)	1.42(1.05-1.89)	-0.7(-0.91–0.49)	81.1(94.01-70.02)	142.44(171.08-117.59)	42.28(36.32-48.74)	27.23(22.67-32.8)	-1.66(-1.75–1.56)
	Severe anemia	Both	0.18(0.28-0.12)	0.11(0.14-0.08)	0.1(0.07-0.16)	0.05(0.03-0.07)	-2.02(-2.34–1.69)	3.66(4.36-3.04)	4.93(6.3-3.84)	1.92(1.6-2.28)	0.93(0.73-1.18)	-2.6(-2.84–2.35)
High-income North America	Anemia	Both	25.01(29.66-21.11)	31.99(37.54-27.06)	8.45(7.1-10.11)	7.91(6.61-9.43)	0.07(-0.05-0.19)	307.85(354.29-268.44)	608.11(706.68-522.74)	84.1(73.52-96.43)	94.81(81.57-109.87)	0.9(0.68-1.12)
	Mild anemia	Both	19.41(23.02-16.37)	25.1(29.31-21.21)	6.52(5.47-7.8)	6.13(5.16-7.25)	0.08(-0.03-0.2)	237.57(273.91-208.13)	465.65(541.18-402.02)	64.94(57.01-74.35)	72.71(62.96-84.32)	0.79(0.59-0.98)
	Moderate anemia	Both	5.45(6.6-4.48)	6.67(8.09-5.52)	1.88(1.54-2.31)	1.72(1.4-2.14)	0(-0.14-0.13)	67.43(80.45-56.61)	136.46(167.31-110.29)	18.39(15.48-21.85)	21.18(17.12-26.04)	1.27(0.94-1.6)
	Severe anemia	Both	0.14(0.18-0.12)	0.22(0.28-0.17)	0.05(0.04-0.06)	0.05(0.04-0.07)	0.75(0.59-0.9)	2.85(3.46-2.31)	5.99(7.66-4.67)	0.77(0.62-0.93)	0.92(0.72-1.17)	1.52(1.18-1.87)
North Africa and Middle East	Anemia	Both	34.25(44.69-26.45)	88.8(113.55-70.17)	9.63(7.76-12.09)	14.52(11.63-18.4)	1.54(1.49-1.59)	248.86(278.29-221.45)	842.75(936.77-757.51)	150.19(134.17-167.77)	207.14(186.1-229.56)	1.16(1.05-1.28)
	Mild anemia	Both	17.37(22.08-13.65)	57.2(72.79-45.49)	5.23(4.26-6.44)	9.42(7.59-11.86)	2.15(2.1-2.21)	144.25(161.66-128.59)	589.9(660.19-526.16)	85.91(76.8-96.28)	144(128.3-160.75)	1.81(1.68-1.93)
	Moderate anemia	Both	15.94(21.31-11.89)	30.11(39.81-23.05)	4.14(3.25-5.41)	4.86(3.75-6.38)	0.66(0.61-0.72)	96.36(107.72-85.68)	239.76(267.5-213.92)	58.69(52.15-65.54)	59.74(53.36-66.55)	0.18(0.04-0.31)
	Severe anemia	Both	0.93(1.25-0.7)	1.49(2.1-1.06)	0.25(0.19-0.33)	0.24(0.17-0.34)	0.07(-0.06-0.19)	8.26(9.37-7.27)	13.09(14.9-11.52)	5.59(4.89-6.33)	3.4(2.98-3.9)	-1.59(-1.65–1.52)
Oceania	Anemia	Both	0.35(0.6-0.19)	0.81(1.57-0.42)	4.43(2.63-7.34)	5.37(2.96-10.05)	0.71(0.64-0.77)	3.31(3.78-2.89)	10.17(11.54-8.91)	108.84(96.11-123.19)	147.62(130.09-166.4)	0.94(0.89-0.98)
	Mild anemia	Both	0.13(0.21-0.08)	0.33(0.61-0.19)	1.74(1.15-2.67)	2.33(1.39-4.08)	1.07(1.02-1.12)	1.91(2.18-1.66)	6.12(6.98-5.32)	63.79(55.95-72.45)	90.27(78.76-103)	1.04(0.98-1.1)
	Moderate anemia	Both	0.2(0.37-0.11)	0.44(0.88-0.22)	2.46(1.36-4.35)	2.82(1.45-5.5)	0.48(0.4-0.57)	1.27(1.47-1.09)	3.72(4.25-3.21)	40.89(35.9-46.6)	52.61(46.06-59.93)	0.81(0.78-0.85)
	Severe anemia	Both	0.02(0.04-0.01)	0.03(0.07-0.02)	0.22(0.12-0.44)	0.22(0.1-0.46)	0.02(-0.13-0.17)	0.13(0.16-0.11)	0.33(0.41-0.27)	4.15(3.47-4.93)	4.74(3.9-5.7)	0.47(0.44-0.5)
South Asia	Anemia	Both	85.54(104.8-69.68)	160.99(196.32-132.81)	7.65(6.35-9.23)	8.72(7.27-10.52)	1.18(0.93-1.43)	618.71(692.62-549.79)	1755.26(1957.9-1565.23)	108.21(96.41-121.2)	128.22(114.72-142.85)	0.56(0.43-0.69)
	Mild anemia	Both	28.06(34.41-23.03)	67.82(82.98-56.06)	2.67(2.22-3.21)	3.62(3.03-4.4)	1.96(1.65-2.26)	190.75(214.9-169.96)	624.48(698.97-554.97)	30.51(27.12-34.25)	44.13(39.46-49.44)	1.42(1.32-1.52)
	Moderate anemia	Both	49.62(61.6-39.93)	84.58(102.94-69.27)	4.28(3.52-5.2)	4.62(3.82-5.58)	0.88(0.66-1.1)	343.53(383.28-304.63)	964.21(1076.78-857.67)	61.18(54.39-68.49)	71.36(63.63-79.38)	0.48(0.33-0.62)
	Severe anemia	Both	7.86(9.92-6.26)	8.59(10.74-6.98)	0.7(0.57-0.86)	0.48(0.4-0.6)	-0.6(-0.84–0.36)	84.43(94.84-74.77)	166.56(188.29-148.13)	16.52(14.6-18.57)	12.73(11.37-14.35)	-1.12(-1.34–0.89)
Southeast Asia	Anemia	Both	34.25(44.58-26.35)	48.61(61.13-39.71)	6.53(5.12-8.33)	7.22(5.87-9.16)	0.74(0.47-1.02)	280.13(313.79-250.14)	945.88(1058.95-843.75)	108.92(96.9-121.75)	163(145.19-181.59)	1.36(1.33-1.39)
	Mild anemia	Both	16.1(20.88-12.49)	29.59(36.24-24.42)	3.16(2.5-4.01)	4.32(3.57-5.29)	1.6(1.27-1.94)	136.91(153.48-121.63)	571.36(640-508.97)	50.74(44.98-56.91)	96.36(85.95-107.68)	2.25(2.19-2.31)
	Moderate anemia	Both	17.13(22.65-12.89)	18.27(24.24-14.41)	3.17(2.43-4.16)	2.8(2.17-3.75)	-0.21(-0.43-0.01)	131.62(147.87-116.83)	354.64(401.59-312.79)	53.24(47.19-59.71)	63.05(55.68-71.41)	0.49(0.45-0.52)
	Severe anemia	Both	1.01(1.36-0.75)	0.75(0.98-0.6)	0.19(0.15-0.25)	0.11(0.09-0.15)	-1.56(-1.76–1.37)	11.6(13.19-10.13)	19.87(23.12-17.14)	4.94(4.34-5.65)	3.6(3.12-4.19)	-1.19(-1.25–1.12)
Southern Latin America	Anemia	Both	3.89(6.31-2.43)	5.03(8.27-3.19)	7.88(5-12.61)	7.38(4.56-12.61)	0.18(-0.04-0.4)	35.13(40.08-30.44)	71.71(82.84-62.6)	77.93(67.89-88.37)	84.5(73.89-97.63)	0.24(0.18-0.3)
	Mild anemia	Both	2.6(4.16-1.68)	3.75(5.99-2.43)	5.29(3.46-8.38)	5.44(3.42-9.02)	0.5(0.25-0.76)	23.53(27.1-20.4)	52.75(60.71-46.16)	51.44(44.92-58.86)	62.31(54.58-71.75)	0.62(0.53-0.7)
	Moderate anemia	Both	1.24(2.19-0.71)	1.24(2.17-0.74)	2.48(1.46-4.3)	1.89(1.08-3.38)	-0.59(-0.75–0.44)	10.88(12.74-9.08)	17.9(21.71-14.71)	24.77(20.78-28.74)	20.95(17.32-25.36)	-0.61(-0.63–0.59)
	Severe anemia	Both	0.05(0.09-0.03)	0.04(0.07-0.03)	0.11(0.07-0.17)	0.06(0.04-0.1)	-1.42(-1.61–1.22)	0.73(0.9-0.58)	1.07(1.35-0.84)	1.72(1.37-2.1)	1.24(0.98-1.57)	-1.12(-1.15–1.09)
Southern Sub-Saharan Africa	Anemia	Both	4.82(6.8-3.55)	8.81(12.73-6.6)	9.06(7.04-12.22)	10.94(8.3-15.75)	0.85(0.61-1.1)	24.22(27.27-21.52)	62.02(69.34-55.51)	87.59(77.47-98.98)	114.57(102.61-128.12)	0.82(0.63-1.02)
	Mild anemia	Both	2.46(3.41-1.83)	4.99(6.91-3.79)	4.83(3.75-6.39)	6.24(4.79-8.52)	1.14(0.83-1.44)	13.47(15.25-11.85)	37.25(41.55-33.22)	48.75(42.5-55.13)	68.51(60.93-76.32)	1.05(0.76-1.35)
	Moderate anemia	Both	2.2(3.28-1.57)	3.58(5.51-2.58)	3.92(2.93-5.53)	4.4(3.21-6.7)	0.54(0.36-0.72)	9.81(11.12-8.6)	22.82(25.99-20.16)	35.44(31.27-40.34)	42.43(37.5-48.45)	0.54(0.46-0.62)
	Severe anemia	Both	0.17(0.23-0.12)	0.24(0.34-0.18)	0.32(0.24-0.43)	0.3(0.23-0.41)	0.03(-0.04-0.1)	0.95(1.13-0.79)	1.96(2.3-1.67)	3.4(2.85-4.04)	3.63(3.09-4.27)	0.2(0.06-0.35)
Tropical Latin America	Anemia	Both	13.06(17.28-10.05)	30.1(37.98-23.76)	8.37(6.59-10.75)	12.97(10.16-16.46)	1.31(1.08-1.54)	102.39(116.5-89.53)	275.79(317.87-237.81)	111.44(97.57-126.73)	114.35(99.01-131.62)	-0.09(-0.16–0.02)
	Mild anemia	Both	6.38(8.28-4.93)	17.53(21.86-14.02)	4.23(3.34-5.33)	7.44(5.91-9.32)	1.66(1.35-1.98)	57.4(65.82-49.66)	172.75(199.99-148.61)	61.82(53.39-70.76)	71.44(61.69-82.5)	0.3(0.23-0.38)
	Moderate anemia	Both	6.18(8.56-4.61)	11.88(15.31-9.09)	3.82(2.94-5.13)	5.23(3.96-6.81)	0.97(0.82-1.12)	41.22(47.65-35.27)	96.05(113.21-80.69)	45.36(38.86-52.49)	39.96(33.6-47.2)	-0.59(-0.66–0.53)
	Severe anemia	Both	0.5(0.73-0.34)	0.68(0.94-0.5)	0.31(0.22-0.44)	0.29(0.21-0.41)	-0.29(-0.42–0.17)	3.78(4.76-3)	6.99(9.07-5.36)	4.26(3.4-5.37)	2.95(2.25-3.8)	-1.39(-1.45–1.33)
Western Europe	Anemia	Both	15.21(18.51-12.63)	18.85(22.88-15.66)	3.72(3.09-4.62)	3.76(3.08-4.62)	0.59(0.32-0.88)	308.38(351.28-272.95)	398.07(453.93-352.58)	51.23(45.64-57.99)	39.79(35.23-45.5)	-1.01(-1.08–0.94)
	Mild anemia	Both	12.17(14.81-10.11)	15.68(19.08-12.99)	2.93(2.43-3.62)	3.07(2.53-3.77)	0.75(0.45-1.04)	238.27(271.48-210.57)	327.08(375.01-289.28)	39.65(35.14-45.01)	32.89(29.03-37.57)	-0.78(-0.84–0.73)
	Moderate anemia	Both	2.96(3.66-2.43)	3.1(3.8-2.55)	0.77(0.62-0.99)	0.67(0.53-0.84)	-0.02(-0.25-0.2)	67.2(77.71-58.76)	68.49(79.17-59.07)	11.1(9.79-12.7)	6.67(5.81-7.64)	-1.91(-2.04–1.78)
	Severe anemia	Both	0.07(0.09-0.06)	0.07(0.09-0.06)	0.02(0.01-0.02)	0.01(0.01-0.02)	-0.36(-0.62–0.1)	2.91(3.44-2.45)	2.5(3.01-2.09)	0.48(0.41-0.56)	0.23(0.2-0.28)	-2.59(-2.75–2.42)
Western Sub-Saharan Africa	Anemia	Both	22.45(27.17-18.15)	76.51(92.44-62.5)	11.65(9.62-13.77)	16.62(13.8-19.68)	1.67(1.51-1.83)	72.69(80.91-65.08)	216.2(240.15-193.53)	83.47(74.68-93.19)	117.37(104.51-130.71)	1.3(1.21-1.4)
	Mild anemia	Both	8.81(10.5-7.21)	32.4(39.08-26.55)	5.26(4.37-6.21)	7.88(6.57-9.36)	1.91(1.73-2.1)	37.2(41.44-33.01)	119.65(132.89-107.1)	41.88(37.25-47.09)	64.8(57.73-72.44)	1.66(1.52-1.79)
	Moderate anemia	Both	11.87(14.63-9.43)	39.77(48.64-32.02)	5.67(4.61-6.81)	7.97(6.56-9.57)	1.59(1.44-1.73)	33.06(37.22-29.32)	90.96(101.59-80.79)	38.74(34.32-43.59)	49.56(43.7-55.38)	0.97(0.89-1.04)
	Severe anemia	Both	1.77(2.24-1.39)	4.34(5.56-3.39)	0.72(0.58-0.9)	0.77(0.61-0.96)	0.48(0.34-0.63)	2.44(2.77-2.14)	5.58(6.39-4.87)	2.85(2.49-3.27)	3.01(2.61-3.49)	0.26(0.16-0.36)

ASPR, age-standardized prevalence rate; EAPC, estimated annual percentage change; UI, uncertainty interval.

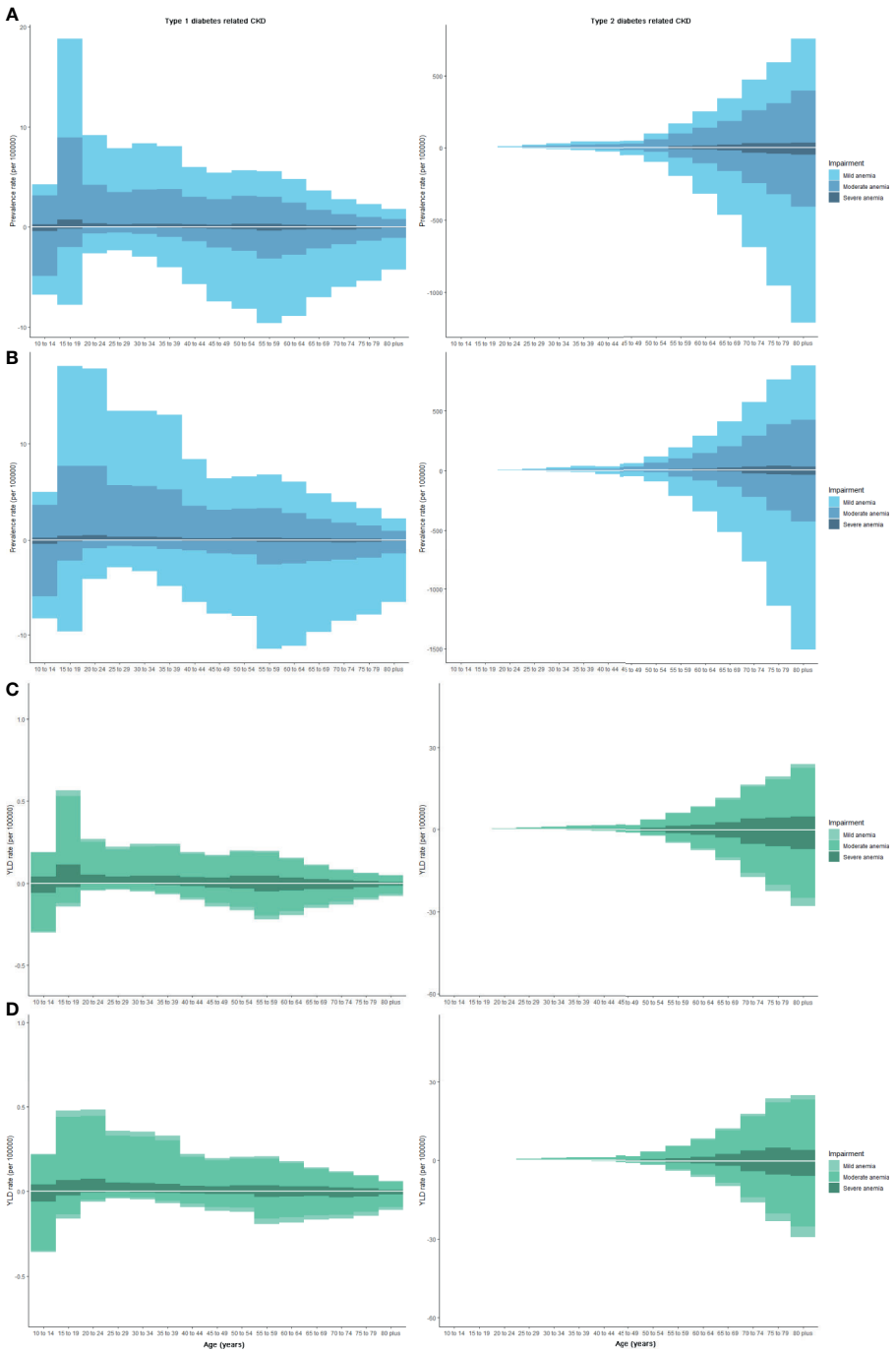

FIGURE 5 | The prevalence and YLD rate of CKD-DM-related anemia at various age subgroups by gender. Each column represents the prevalence or YLD rate (per 100,000 people) of CKD-DM-related anemia (Three grades: mild, moderate, and severe). The upper column in each age-group is data for females, and the below column is for males. **(A)** Prevalence in 1990; **(B)** Prevalence in 2019; **(C)** YLD rate in 1990; **(D)** YLD rate in 2019. CKD, chronic kidney disease; YLD, years lived with disability.

the general population (24). Nevertheless, not all patients with CKD-DM could receive renal replacement therapy, and 78% of patients lived in low- and middle-income countries, where resources, availability of dialysis, and kidney transplants were limited (25). However, the difference was not fully attributed to medical convenience. ASIR and ASPR of CKD-T1DM increased only in Eastern Europe, with the lowest ASDR of CKD-T2DM. ASIR of CKD-T1DM decreased only in High-income North America, but ASDR and DALY rate increased faster there. ASDR and DALY rate decreased faster in High-income Asia Pacific and

East Asia. The difference of CKD-DM between regions might result from the gap in genetic, ethnic, and dietary risk factors.

Patients with CKD-T1DM- and CKD-T2DM-related anemia had doubled over the past 30 years. Anemia is a common complication of CKD. Among all causes of anemia, malaria, schistosomiasis, and CKD-related anemia have been on the rise (26). However, the severity and type of anemia were various among regions. The higher the SDI value, the lower the increasing rate of anemia-related ASPR, mirroring the gap in life and medical convenience among different SDI quintiles. Furthermore, there was 40% of the population with anemia in Ghana (27), a country in Western Sub-Saharan Africa, resulting from iron deficiency, hemoglobinopathies, micronutrient deficiency, and inflammation (26, 28). In addition, we should attach importance to the fast increase of ASPR in Central Asia. Conversely, in Western Europe, High-income Asia Pacific, and East Asia, ASPR of CKD-T2DM-related anemia decreased sharply, and the reasons should be further evaluated. In Austria, the incidence of type 1 diabetes is increasing in children aged 5 to 14 years (29). Alarmingly, for CKD-T1DM-related anemia, patients aged of 10–14 years mainly suffered moderate anemia, more severe than other age-groups, which we should pay attention to.

In many cases, the burden of CKD-DM is determined by various factors, which caused gaps in the CKD prevention and management capabilities worldwide (30). Our results reflected a shift of CKD-T1DM burden from high to low SDI quintile, but the ASDR and DALY rate of CKD-DM increased faster in high SDI quintile, which was not fully attributed to medical environment and renal replacement therapies (31). Global burden of CKD-DM was concentrated in middle SDI quintiles, especially in developing countries (20). Additionally, ASIR of CKD-DM increased with SDI value, revealing racial differences in disease susceptibility and medical disparities (32, 33). The variation in CKD-DM epidemiology reflects huge regional inequities in preventive care (34). White European individuals were reported to have a higher prevalence of CKD-T1DM (35). Race influenced mortality in patients with type 2 diabetes and multiple chronic conditions (36). Some studies explained it by economic inequality, socioeconomic status, and segregation (37–39).

Understanding the burden of CKD-DM in various countries benefited equal kidney health. China, India, and the USA carrying high disease burden for CKD-DM might partly be owing to their high populations. Notably, China had the lowest ASIR of CKD-T1DM. ASPR of CKD-T1DM was higher in Russia, Canada, and Mongolia. This was partially attributed to high prevalence of type 2 diabetes, improvements on CKD screening (40), and the relatively stagnant progress in addressing CKD-DM burden.

Although aging and population growth contributed to the increased burden of CKD-DM, risk factors such as diet and metabolism were involved. A study on children stated that type 1 diabetes was associated with younger age at ESRD onset, whereas type 2 diabetes was related to a higher mortality rate (41). The presence of diabetic nephropathy was associated with age, duration of diabetes, and poor glycemic control (42).

Almost one in five CKDs was caused by diabetes (10). Moreover, less than half of the patients were tested for urinary albumin, an early marker of kidney disease caused by diabetes (43). Many countries still lack a well-trained team of kidney experts and universal access to primary health care and renal replacement therapy. Screening for kidney function in diabetic patients as well as raising awareness are necessary for the early detection of CKD. Reducing the burden of CKD-DM should be reflected in the government's health priorities and resource allocation measures, focusing on prevention, early control, and delayed progress.

Some inevitable limitations should be taken into consideration in the interpretation of our findings. The GBD study estimated the burden of CKD by relying on statistical methods and predicted covariant values. GBD data come from census, disease registration, household survey, health service usage, air pollution monitoring, disease notification civil registration and vital statistics, and other sources. High-quality results were based on well-established medical registration systems in some countries, such as China, USA, India, Australia, UK, Russia, and so on. GBD 2019 location hierarchy includes all WHO member states. Large, high-quality, population-based studies of CKD are scarce in some countries or territories, such as Cook Islands, Niue, Vatican City, Liechtenstein, Order of Malta, Palestine. There was inevitable information bias of primary data in those districts. Therefore, when specific data were applied to countries or territories that are not members of the World Health Organization, and areas with underdeveloped medical systems, the findings need to be interpreted with caution. Due to the limited data, we cannot further investigate the burden of CKD-DM at different stages. A greater investment is still needed to improve vital registration and data collection in developing countries. Despite these limitations, the findings from this analysis add novel knowledge on the global burden of CKD-DM.

CONCLUSION

From 1990 to 2019, the increasing burden of CKD-DM varied among regions and countries. All ASRs of CKD-T2DM exhibited upward trends, and from the age of 50, all rates were higher in males than females. ASIR of CKD-DM increased with SDI value. Middle SDI quintile accounted for the majority burden of CKD-DM worldwide. Asia carried the heaviest burden of CKD-DM, especially in South and East. The three countries with the highest burden of CKD-DM were China, the United States, and India. CKD-T2DM patients with anemia were mainly in mild to moderate grade for females, and in mild grade for males. Anemia-related YLD was mainly in moderate grade. These findings could help guide the epidemiological monitoring of this disease and prioritize the most appropriate health interventions.

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 Ethics Committee of the Second Affiliated Hospital, College of Medicine, Xi'an Jiaotong University. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. ZD, JG, and YD designed the study. YD, YW, MW, and SY conducted the initial searches. YiZ and XD collected the data and verified the accuracy of the data. DX and YuZ contributed to data interpretation. YD, ZZ, and DZ performed the statistical analysis and visualization. YD wrote and revised the manuscript.

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The authors analyzed and interpreted the data for this manuscript. All aspects of manuscript writing were carried out by the authors.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.672350/full#supplementary-material

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Sex Differences in Biopsy-Confirmed Diabetic Kidney Disease

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Background: To investigate the association between sex differences and end-stage kidney disease (ESKD) in patients with biopsy-confirmed diabetic kidney disease (DKD).

Method: We performed a retrospective cohort study. A total of 336 patients with biopsy-confirmed DKD who were followed up for at least 12 months were enrolled. Baseline clinical and pathological data at the time of biopsy were collected. ESKD was defined by an estimated glomerular filtration rate of <15 ml/min/1.73 m^2 or initiation of renal replacement therapy. The association between sex differences and ESKD was assessed using the log-rank test and Cox regression.

Result: There were 239 (71%) male and 97 (29%) female patients in our cohort. Female patients had higher systolic blood pressure, total cholesterol and low-density lipoprotein cholesterol levels compared with male. There were a lower proportion of female patients in the very high risk grade according to the chronic kidney disease categories (37% of female vs. 44% of male). During a median follow-up time of 20 months, 101 (57.7%) male and 43 (44.3%) female entered into ESKD, with no significant difference by the log-rank test (P >0.05). Univariate [male: hazard ratio (HR) [95% confidence interval (CI)], 1.005, (0.702–1.439)] and multivariable ([male: HR (95%CI), 1.164, (0.675–2.007)]. Cox regression further showed that sex difference was not significantly associated with ESKD.

Conclusion: Female patients had the higher systolic blood pressure, total cholesterol, LDL-C, compared with male patients. However, there was no significant association observed between sex difference and ESKD in our study.

Keywords: sex differences, diabetic kidney disease, end stage kidney disease, risk factors, type 2 diabetes

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INTRODUCTION

Diabetic kidney disease (DKD) is one of the most common microvascular complications of diabetes. Despite improvements of management in basic research and clinical practice, DKD remains the leading cause of end-stage kidney disease (ESKD) worldwide (1, 2). In order to slow down the progression of DKD, recognizing patients with a high risk at an early stage is important. Sex differences have been taken into account in development or progression in several diseases such as diabetes (3), chronic kidney disease (CKD) (4), heart failure (5), and neuropsychiatric disorders (6). Recently, a study from the Chronic Renal Insufficiency Cohort that included 3,939 adults (half of

them had diabetes) showed that male patients had the higher risk of CKD progression and death compared with female patients (4). Similarly, another large meta-analysis reported that males with CKD showed a more rapid decline in renal function than which in females, however, only patients with nondiabetic CKD were analyzed in that study (7).

The association between sex differences and the incidence or progression of DKD has been investigated in several studies, but not been well established with disparate conclusions (8). Different ethnic cohorts, age, type of diabetes and study designs can all cause the contradictory results. Moreover, most of the patients did not receive a kidney biopsy in these previous studies. Differences between DKD and nondiabetic kidney diseases greatly contribute to the challenges of understanding diabetic complications. Patients with nondiabetic kidney diseases might have confounded the results in previous study.

Therefore, in the current study, we aimed to investigate sex differences of clinical and pathological characteristics in patients with biopsy-confirmed DKD. We also aimed to evaluate the association between sex difference and ESKD.

METHOD

Study Design and Patients

We performed a retrospective cohort study. The study was approved by the ethics committee of West China Hospital of Sichuan University and all patients have signed a written informed consent form.

Patients with biopsy-confirmed DKD from January 2010 to December 2018 in our hospital were reviewed. Baseline data were collected from the hospital information system at the time patients received a kidney biopsy. The inclusion criteria were as follows: a. type 2 diabetes; b. biopsy-confirmed DKD; and c. follow-up for longer than 12 months (patients who developed ESKD in 12 months were also included). Type 2 diabetes was diagnosed in accordance with the 2018 American Diabetes Association criteria (9). Renal pathological classifications were based on the Renal Pathology Society in 2010 (10) by at least two professional pathologists. ESKD was defined as initiation of renal replacement therapy or eGFR less than 15 ml/min/1.73 m^2.

Statistical Analysis

Continuous variables were described as mean ± standard deviation (SD) or median and quartiles on the basis of a normality test. Categorical variables were presented as counts with ratios. Differences of baseline data between male and female patients were evaluated appropriately by the Student's t test or the Mann–Whitney test. The prognosis of the kidney was compared by the log-rank test and shown using the Kaplan–Meier curve. Univariate and multivariate Cox analysis were applied to determine the risk factors of ESKD. All analyses were conducted using SPSS software 22.0 and GraphPad Prism 7.0. A two-sided *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Baseline Clinical and Pathological Characteristics

A total of 336 patients were enrolled in the study. Baseline clinical and pathological characteristics are shown in **Table 1**. Briefly, the mean age of patients was 51.7 ± 8.95 years old, 291 (86.6%) patients had hypertension, the median diabetic duration was 96 (36–141) months. The median eGFR was 59 (43–93) ml/min/1.73 m^2 and the median proteinuria was 4.3 (2.0–7.8) g/24 h. There were 239 (71.1%) male and 97 (28.9%) female in our cohort; compared to male, female had the higher level of systolic blood pressure and lipid metabolism. Moreover, a significantly higher proportion of female patients received renin–angiotensin–aldosterone system (RAAS) inhibitors therapy. Male had the higher level of serum creatinine compared with female. There were no significantly differences in age, diastolic blood pressure, the duration of diabetes, the incidence of diabetic retinopathy, blood glucose, proteinuria, triglyceride, medical insurance, insulin use, statins and fibrates use. With regard to pathological lesions, 16 patients had glomerular class I, 77 had class IIa, 33 had class IIb, 153 had class III, and 52 had class IV. However, there were no significant differences in glomerular class, interstitial fibrosis and tubular atrophy (IFTA), interstitial inflammation and arteriolar hyalinosis between male and female patients.

Metabolic Characteristics Between Male and Female Patients

With regard to metabolic characteristics, the body mass index (male vs. female 25.7 (23.2–27.8) kg/m^2 vs. 25.4 (23.2–27.5) kg/m^2, P >0.05) and triglyceride (male vs. female 2.09 ± 1.562 mmol/L vs. 2.45 ± 2.152 mmol/L, P >0.05) were not significant different between male and female. However, compared with male patients, female patients had the significantly higher total cholesterol (male vs. female 4.96 ± 1.45 mmol/L vs. 5.92 ± 1.84 mmol/L, P <0.05), LDL-C (male vs. female 2.93 ± 1.144 mmol/L vs. 3.46 ± 1.470 mmol/L, P <0.05), HDL-C (male vs. female 1.28 ± 0.532 mmol/L vs. 1.49 ± 0.534 mmol/L, P <0.05), but lower uric acid (male vs. female 397 (349–451) mmol/L vs. 354 (311–391) mmol/L, P <0.05).

CKD Risk Categories

To evaluate the risk distribution between male and female patients, we used the CKD category heat map as recommended by the Kidney Disease Improving Global Outcomes (11). Patients were categorized into low risk (green), moderately increased risk (yellow), high risk (orange) and very high risk (red) grades by baseline proteinuria (24 hour-proteinuria of 306 patients were obtained) and eGFR. Those patients in the red category had the highest proteinuria and lowest GFR, and carried highest risk for events of cardiovascular disease, ESKD and mortality. A total of 9% (28/306) of patients were low risk, 21% (65/306) of patients had a moderately increased risk, 27% (84/306) were high risk, and 42% (129/306) were very high risk (**Figure 1A**). As for sex distribution, both approximately 30% of

TABLE 1 | Baseline clinicopathological findings in male and female groups.

Variables	Total (n = 336)	Male (n = 239)	Female (n = 97)	*P* value
Age (years)	51.7 ± 8.95	51.5 ± 8.92	52.1 ± 9.06	>0.05
Body mass index (kg/m^2)	25.7 (23.2–27.7)	25.7 (23.2–27.8)	25.4 (23.2–27.5)	>0.05
Current Smoker (n, %)	107 (32)	103 (43)	4 (4)	<0.001
Hypertension (n, %)	291 (86.6)	202 (84.5)	89 (91.8)	>0.05
Systolic blood pressure (mmHg)	145 ± 23.1	143 ± 22.5	152 ± 23.4	0.001
Diastolic blood pressure (mmHg)	86 ± 13.2	85 ± 12.1	88 ± 15.7	>0.05
Diabetes duration (months)	96 (36–141)	96 (36–144)	96 (36–132)	>0.05
Diabetic retinopathy (n, %)	153 (47) (n = 327)	105 (46) (n = 230)	48 (49) (n = 97)	>0.05
Fasting blood glucose (mmol/L)	8.3 ± 4.16	8.2 ± 3.99	8.6 ± 4.54	>0.05
Glycosylated hemoglobin (%)	7.3 (6.2–8.6)	7.4 (6.3–8.6)	7.2 (6.1–8.4)	>0.05
Serum albumin (g/L)	34.3 ± 7.74	34.7 ± 7.57	33.2 ± 8.08	>0.05
Hemoglobin (g/L)	120.2 ± 27.9	125.4 ± 28.7	107.1 ± 20.7	<0.05
Serum creatinine (umol/L)	119 (80–158)	127 (89–163)	96 (70–137)	<0.001
eGFR (ml/min/1.73 m^2)	59 (43–93)	58 (43–92)	61 (42–94)	>0.05
BUN (mmol/L)	9.1 ± 5.42	9.1 ± 4.01	9.1 ± 7.92	>0.05
Proteinuria (g/24 h)	4.3 (2.0–7.8) (n = 306)	4.3 (2.2–7.8) (n = 214)	4.3 (1.8–7.7) (n = 92)	>0.05
Triglyceride (mmol/L)	2.20 ± 1.757	2.09 ± 1.562	2.45 ± 2.152	>0.05
Total cholesterol (mmol/L)	5.24 ± 1.63	4.96 ± 1.45	5.92 ± 1.84	<0.001
LDL-C (mmol/L)	3.08 ± 1.267	2.93 ± 1.144	3.46 ± 1.470	0.002
HDL-C (mmol/L)	1.34 ± 0.541	1.28 ± 0.532	1.49 ± 0.534	0.001
Uric acid (mmol/L)	383 (337–434)	397 (349–451)	354 (311–391)	<0.001
Medical insurance (n, %)	223 (66.4)	158 (66.1)	65 (67.0)	>0.05
Pathological lesions (n = 331)				
Glomerular class (n = 331)				>0.05
I	16	12	4	
IIa	77	57	20	
IIb	33	25	8	
III	153	04	49	
IV	52	36	16	
Interstitial fibrosis and tubular atrophy				>0.05
0	10	8	2	
1	141	93	48	
2	139	103	36	
3	41	31	10	
Interstitial inflammation			n = 96	>0.05
0	20	17	3	
1	241	161	80	
2	70	57	13	
Arteriolar hyalinosis				>0.05
0	32	23	9	
1	170	125	45	
2	129	87	42	
Use of medications				
RAAS inhibitors (n, %)	267 (79.5)	183 (76.6)	84 (86.6)	0.039
Insulin use (n, %)	240 (71.9)	166 (69.7)	74 (77.1)	>0.05
Statins (n, %)	193 (57.4)	129 (54.0)	64 (66.0)	>0.05
Fibrates (n, %)	15 (4.5)	10 (4.2)	5 (5.2)	>0.05
Follow-up information				
Follow-up duration (months)	20 (14–35)	19 (13–35)	23 (14–36)	>0.05
ESKD (n, %)	144 (57.1)	101 (57.7)	43 (44.3)	>0.05

Data are presented as the mean ± standard or counts and percentages.
eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RAAS, Renin–angiotensin–aldosterone System; ESKD, end stage kidney disease.
A two-tailed p <0.05 was considered statistically significant.

male and female patients had low and moderately increased risks, but more male had a higher risk than female (44% vs. 37%) (**Figure 1B**).

Sex Difference and ESKD

During a median follow-up period of 20 (14–35) months, a total of 144 (57.1%) patients developed ESKD. Specifically, there were 101 (57.7%) male, 18 (52.9%) premenopausal female, and 25 (39.7%) menopausal female suffered from ESKD during the follow-up time. There was no significant difference in kidney survival between male and female, and no difference between premenopausal and menopausal female (**Figure 2**).

To evaluate risk factors of ESKD in patients with DKD, we performed univariate and multivariable Cox regression analyses (**Table 2**). Specifically, the hazard ratio (HR) and 95%

A

				Proteinuria (g/24 hours)		
				P1	P2	P3
				Normal to mildly increased	Moderately increased	Severely increased
				<1	1-3.5	⩾3.5
eGFR (mL/min/1.73m2)	G1	Normal or high decreased	≥90	21	34	32
	G2	Mildly decreased	60-89	7	21	36
	G3a	Mildly to moderately decreased	45-59	7	15	44
	G3b	Moderately to severely decreased	30-44	3	13	36
	G4	Severely decreased	15-29	1	4	27
	G5	Kidney failure	<15	0	0	5

B

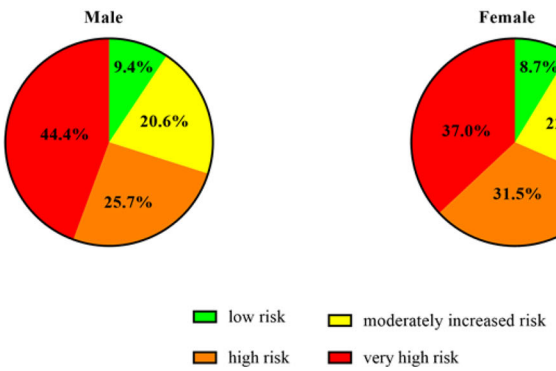

FIGURE 1 | Prognosis of CKD categories and sex. Proteinuria (g/24 hours) of 306 patients were obtained at the baseline. Green, low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk. The digits in **(A)** cells represent the numbers of patients. **(B)** represent the percentage of male and female in different categories.

confidence interval (CI) of male was 1.005 (0.702–1.439, P = 0.978), which indicated there was no association between sex and ESKD. The higher levels of systolic blood pressure, proteinuria, total cholesterol, LDL-C, HDL-C, advanced class of glomerular lesion, IFTA, interstitial inflammation, arteriolar hyalinosis, incidence of diabetic retinopathy, and the lower levels of serum albumin and eGFR were associated with ESKD. Moreover, when we adjusted for essential clinical and pathological indices, sex was still not associated with ESKD (HR and 95% CI, 1.164, 0.675–2.007, P = 0.584). However, a higher levels of interstitial inflammation (HR and 95% CI, 1.705, 1.041–2.791, P = 0.034), and the lower serum albumin (HR and 95% CI, 0.895, 0.858–0.932, P < 0.001) and eGFR (HR and 95% CI, 0.969, 0.959–0.979, P < 0.001) were independently associated with ESKD.

DISCUSSION

DKD has become the leading cause of ESKD, which has led to a heavy economic burden on individuals and countries (2). Therefore, recognizing risk factors of ESKD would be beneficial for to slowing the progression of DKD. The association between sex difference and ESKD in patients with DKD has not been well established. In the current study, the proportion of male was higher than that of female. We also found that male patients had relatively good control of lipid metabolism. However, more male patients were in the high very risk grade of CKD categories at baseline compared with female. However, there was no association between sex difference and ESKD in our study.

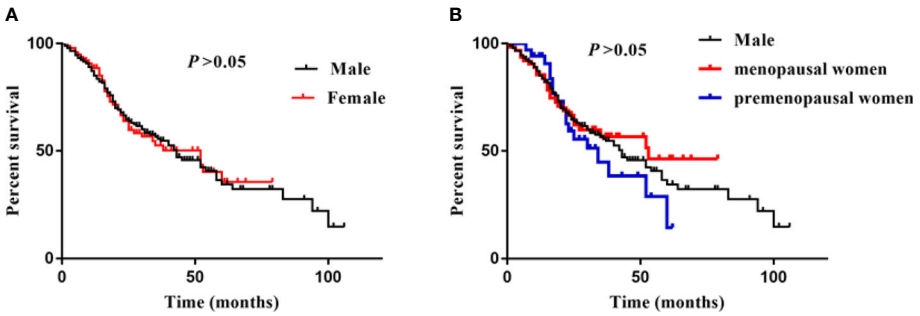

FIGURE 2 | Sex difference and ESKD. **(A)** was showed survival curves of male and female, **(B)** was showed survival curves of male, menopausal/premenopausal women. Log-rank analysis was used to compared the percent survival between male and female. There was no significant difference between male, premenopusal and menopausal female.

TABLE 2 | Risk factors of ESKD.

Variables	HR	95% CI Univariate	*P* value	HR	95% CI Multivariate	*P* value
Male	1.005	0.702–1.439	0.978	1.164	0.675–2.007	0.584
Age	0.991	0.973–1.008	0.303	0.975	0.951–0.999	0.043
Systolic blood pressure	1.007	1.000–1.015	0.045	0.995	0.986–1.005	0.341
Current smokers	0.900	0.634–1.278	0.556	0.850	0.500–1.446	0.549
Diabetes duration	1.002	0.999–1.004	0.171	1.001	0.998–1.005	0.466
HbA1c	0.927	0.842–1.021	0.125	0.955	0.863–1.058	0.381
Diabetic retinopathy	1.876	1.344–2.619	<0.001	1.487	0.948–2.333	0.084
Serum albumin	0.899	0.879–0.920	<0.001	0.895	0.858–0.932	<0.001
eGFR	0.968	0.961–0.975	<0.001	0.969	0.959–0.979	<0.001
Proteinuria	1.104	1.076–1.134	<0.001	1.003	0.944–1.066	0.919
Triglyceride	0.935	0.842–1.039	0.211	0.817	0.598–1.116	0.205
Total cholesterol	1.184	1.075–1.303	0.001	1.606	0.777–3.320	0.201
LDL-C	1.221	1.081–1.378	0.001	0.537	0.260–1.111	0.094
HDL-C	1.320	1.017–1.712	0.037	0.703	0.318–1.552	0.383
Glomerular class	1.758	1.482–2.085	<0.001	1.029	0.782–1.354	0.837
IFTA	1.813	1.456–2.257	<0.001	0.769	0.519–1.139	0.190
Interstitial inflammation	2.938	2.105–4.100	<0.001	1.705	1.041–2.791	0.034
Arteriolar hyalinosis	1.518	1.171–1.967	0.002	1.103	0.771–1.578	0.590

eGFR, estimated glomerular filtration rate; LDL-C, low-density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; IFTA, interstitial fibrosis and tubular atrophy; HR, Hazard ratio; CI, confidence interval. Univariate and multivariate indicated that sex was not associated with ESKD.
A two-tailed p < 0.05 was considered statistically significant.

Increasing studies have investigated the effect of sex differences on DKD development and progression, however, but different cohorts have reported conflicting findings. In studies that enrolled patients with type 2 diabetes, it seems that more results indicated female has greater risk of DKD progression (8). A study from Japan (12) (247 male and 97 female) showed that the mean annual decline in the eGFR was 3.5% in female and 2.0% per year in male. However, this study only enrolled patients with diabetes or those at the early stage of CKD (mean eGFR >90 ml/min/1.73 m^2, only 28.5% of patients had proteinuria). Similarly, several studies showed that African American, Hispanic and Pima Indian female had a higher risk of DKD and disease progression (13–15). Nevertheless, another prospective observational study (227 male and 60 female) enrolled patients with type 2 diabetes and persistent macroalbuminuria (≥300 mg/24 h) and showed that sex difference had no association with DKD progression (16). This previous finding is similar to our results. In our study, the ratio of male and female (approximately 2.5) was consistent with previous studies, but patients with the lower eGFR and greater proteinuria. Moreover, studies have found that the effect of sex is less apparent in DKD than in non-DKD (17, 18). Our patients with DKD were all diagnosed by a kidney biopsy, which excluded non-DKD, and this could explain the results.

The recognition of underlying mechanism of sex differences in diseases remains limited. Sex hormones are considered to be the main driver of sex disparities in the incidence and progression of CKD. A meta-analysis that included 11,345 patients clearly indicated that male was associated with a faster progression of nondiabetic CKD (7). However, this renoprotective effect was only evident in premenopausal female (19, 20). Once patients suffer from diabetes, the renoprotective effect of female is generally considered lost, even in premenopausal female (21). Accumulating evidence

suggests that patients with diabetes have unbalanced levels of sex hormones, where expression of estradiol is decreased, but testosterone is increased, in female with diabetes (22, 23). Moreover estrogen replacement alleviates pathological lesions in animal DKD models (24–26), and can even attenuate proteinuria and improve creatinine clearance in postmenopausal female with diabetes (27). In our study, most of female were during perimenopause which worsened the imbalance of hormones. This could also explain why there was no significantly difference in kidney survival among premenopausal, menopausal female and male in our cohort.

There are other possible mechanisms contribute to sex differences. Studies have suggested that more adolescent female with diabetes had hyperfiltration in the early stage of DKD (28, 29). Additionally, the higher baseline total cholesterol and LDL-C of our female patients, which was consistent with a cohort from Australia (30), also increased the risk of hyperfiltration. Hyperfiltration traditionally indicates a poor kidney prognosis, but a recent study from the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) followed up 446 patients with type 1 diabetes for longer than 20 years found that early hyperfiltration was not associated with decreased renal dysfunction (31). Therefore, although female patients with diabetes are more likely to have hyperfiltration, this does not affect kidney prognosis. The expression and mechanism of several therapeutic targets had been found different between male and female. Specifically, some studies have observed that male had the higher expression of ANG II (32, 33), and ANG II is recognized to mediate renal inflammation (34). Additionally, the expressions of sodium-glucose co-transporters (SGLT) 1 and SGLT2 have been found higher in female rats than in male rats (35, 36). A recent meta-analysis also showed that a reduction in major adverse cardiac events with SGLT2 inhibitors was less in female with diabetes compared with male with diabetes (37). The underlying mechanisms of these differences remain unclear, but it is worthy to be further investigated to provide individual therapy and improve prognosis of patients with diabetes.

There were several limitations should be addressed. First, this was a retrospective cohort study, and we only observed the relationship between sex differences and kidney prognosis. therefore, prospective studies are warranted to determine the underlying causative relationship. Second, our study only included Chinese patients, various genetic backgrounds might have affected our results. Third, we had no opportunity to evaluate the levels of sex hormones at baseline owing to the study design. Fourth, the sample size was limited and patients were in a relatively severe disease stage because we only enrolled patients with biopsy-confirmed DKD. Therefore, further prospective and large sample size DKD cohorts are required to investigate the issue.

CONCLUSION

In patients with biopsy-confirmed DKD, female patients had the higher systolic blood pressure, total cholesterol, LDL-C levels, compared with male patients. However, there was no significant association was observed between sex difference and ESKD in our study.

PERSPECTIVES AND SIGNIFICANCE

Sex differences play an important role in many diseases including cancers or chronic diseases. However, the association between sex differences and the incidence or progression of DKD has not been well established with disparate conclusions. Therefore, we investigate the issue in patients with biopsy-confirmed DKD. We found that female patients had the higher systolic blood pressure, total cholesterol, LDL-C levels. However, there was no association between sex difference and ESKD in our study. The study provides relatively strong evidence to illustrate the associations.

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 ethics committee of West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YTW, JZ, and FL planed, analyzed and wrote the manuscript. JLZ, YCW, RZ, and HR collected data, check analysis and gave some suggestions. MC revised the manuscript and gave lot of suggestions. All authors contributed to the article and approved the submitted version.

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Diffusion Tensor Imaging in Rat Models of Preclinical Diabetic Nephropathy: A Preliminary Study

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Purpose: This study aimed to investigate the value of diffusion tensor imaging to assess renal injury in a rat model of preclinical diabetic nephropathy.

Methods: Twenty-eight male Sprague Dawley rats were divided into two groups: the normal control (NC) group of 10 rats and the diabetic nephropathy (DN) group of 18 rats. Eight weeks after diabetes induction by streptozotocin, 3.0-T magnetic resonance (MR) imaging (b = 0 and 600 s/mm^2, 15 diffusion directions) using a 32-channel knee coil was performed. After MR imaging, we measured serum creatinine, and collected double kidney tissues for pathology. The apparent diffusion coefficients(ADC) and fractional anisotropy(FA) values of the renal cortex and medulla were calculated for all kidneys. Physiological parameters, laboratory parameters, and imaging results were compared between the two groups.

Results: All DN group animals developed hyperglycemia, polyuria, and emaciation. Serum creatinine was not significantly different between the groups ($P > 0.05$). Urinary albumin at 2, 4, and 8 weeks was higher in the DN group than in the NC group but <20 μg/min ($P < 0.05$). Pathologically, renal damage in the DN rats was observed. The ADC value was significantly increased in DN animals in the cortex (1.75×10^{-3}mm^2/s),medulla (1.53×10^{-3}mm^2/s)compared with NC group(cortex, 1.52×10^{-3}mm^2/s; medulla,1.35×10^{-3}mm^2/s). The FA value was significantly reduced in DN animals in the cortex (0.21),medulla(0.25)compared with NC group(cortex,0.26;medulla,0.3).

Conclusions: Increased apparent diffusion coefficients and decreased fractional anisotropy values on diffusion tensor imaging were associated with preclinical DN. Diffusion tensor imaging may be useful in early, non-invasive, quantitative detection, and therapy monitoring of DN.

Keywords: diffusion tensor imaging, kidney, diabetic nephropathy, apparent diffusion coefficient, fractional anisotropy

INTRODUCTION

As a serious microvascular complication of diabetes mellitus, diabetic nephropathy (DN) is one of the major causes of end-stage renal disease (1, 2) and can induce structural changes in the kidney, including tubular dilatation, thickening of the glomerular basement membrane, and nodular and diffuse glomerulosclerosis (3). However, pathological changes are not used for therapy monitoring because biopsy is invasive and prone to sampling errors. At present, the earliest clinical evidence of DN is microalbuminuria, but in preclinical DN, excretion of urinary albumin can be within the normal range, and not all patients with microalbuminuria develop DN (4). Therefore, identification of a reliable non-invasive imaging marker for monitoring the treatment and prognosis of DN is necessary. Diffusion is the random and irregular movement of molecules. It is an important physiological activity of human body and one of the transport modes of substances in the body. In normal tissue, diffusion is rarely limited. However, in the pathological state, due to the influence of various factors, the diffusion will be limited. In human tissues, the movement of water molecules varies in different directions due to the infulence of the celluar structure of the tissue. Diffusion tensor imaging (DTI) can apply motion-sensitive gradients in at least six directions to noninvasively evaluate the diffuse motion of water molecules. DTI (5, 6) is a promising non-invasive technique that can assess renal function and pathology by qualitatively and quantitatively imaging three-dimensional diffusion of water molecules. DTI can not only describe the direction of diffusion of water molecules in tissues by fractional anisotropy (FA), but also describe the displacement degree of water molecules in tissues in the direction of diffusion sensitive gradient by apparent diffusion coefficient (ADC). The characteristics of tissues and organs can be quantitatively reflected by ADC and FA (7).In recent years, more and more studies have used DTI to evaluate diabetic nephropathy, and found that it has potential clinical value (8, 9). Lu (10) have suggested that the apparent diffusion coefficient and fractional anisotropy value may be viable imaging biomarkers in DTI that can reflect the pathological progression of DN. The purpose of this study was to investigate whether the apparent diffusion coefficient (ADC) and fractional anisotropy (FA) value can be used to quantitatively evaluate renal function changes in preclinical DN and provide a non-invasive, visual, and accurate imaging method for the diagnosis of DN.

MATERIALS AND METHODS

Diabetic Nephropathy Model Rats

Efforts such as improving comfortable feeding environment, minimizing the times of invasive procedures, pre-operation training of animals and euthanasia were made to minimize the suffering. The animal experiments were performed in accordance with the China Laboratory animal-Guideline for ethical review of animal welfare and were approved by the ethics committee of our institution (2019048).

Twenty-eight male Sprague Dawley rats weighing 498.5 ± 54.3 g(provided by Chengdu Dashuo experimental animal co., LTD, China) were randomly divided into two groups: the diabetic nephropathy (DN) group with 18 rats and the normal control (NC) group with 10 rats. DN group used streptozotocin (Sigma, USA) to establish diabetic nephropathy model (11, 12). 500mg streptozotocin (STZ) was dissolved in 50ml sodium citrate buffer (0.1mol/L, pH4.5) (Beijing Solaibao Technology Co., LTD, China) to prepare STZ solution. The whole operation was carried out under the conditions of dark and ice bath. Because the prepared solution is very unstable, it needs to be used and prepared now, and the injection should be completed within 30 minutes. A dose of 40 mg/kg STZ solution in the DN group was injected intraperitoneally to establish the DN model after 10 weeks of high-glucose and high-fat diet (provided by Chengdu Dashuo experimental animal co., LTD, China). The NC group rats were given a normal diet. General parameters, including food and water intake, body weight, as well as 24-hour urine volume collected by metabolic cage (Purchased from Shanghai Jianyi Instrument Equipment Co., LTD, China) of all animals were monitored regularly. Serum creatinine (Nanjing Jiancheng Biological Engineering Institute, China) and urinary albumin (Nanjing Jiancheng Biological Engineering Institute, China) at 2, 4, and 8 weeks levels were quantified according to the manufaturer's guidelines. Blood samples from the caudal vein were taken 72 hours later to measure fasting serum glucose (Jinwen, China). According to the diagnostic criteria of diabetes mellitus (13), a rat fasting serum glucose level of >16.7 mmol/L and symptoms, such as increased drinking water, diet, urine volume, and loss of weight, are indicators that the diabetes model has been established.

MR Protocol and Data Collecting

All animals were scanned at 8 weeks after diabetes induction by using a 3.0-T scanner (Discovery MR 750; GE, USA) with a 32-channel knee coil. To restrain the animals during MR scanning, 10% chloral hydrate(2 ml/kg) (Dalian Meilun Biotechnology Co., LTD, China) was used. There were no signs of peritonitis in the rats after treated with chloral hydrate. T1-weighted axial images were acquired: repetition time/echo time, 360/9.5 ms; field of view (FOV), 80 × 80 mm^2; matrix, 160 × 128; number of excitations (NEX), 4; and thickness/interval, 2.0/0.2. The spin-echo DTI sequence parameters were as follows: repetition time/echo time, 4000/89 ms; FOV, 80 × 80 mm^2; matrix, 160 × 128; NEX, 4; thickness/interval, 2.0/0.2; b-values, 0 and 600 s/mm^2; and diffusion directions, 15. Images were analyzed by two readers blinded to the pathological and laboratory results on a post-processing workstation (AW4. 5). ADC and FA values were obtained by the two experienced radiologists by drawing six regions of interest (ROIs) with sizes of 2 to 3 mm^2 in renal cortex and medulla. When drawing the ROI, we select multiple ROIs at multiple levels above and below the renal hilum for data

measurement, and avoid artifacts and vascular expectations to ensure the accuracy and authenticity of the data measurement. The mean ADC and FA value were calculated for statistical analysis. Images with obvious artifacts will be excluded.In addition, the rats with positive urine albumin should also be eliminated.

Histopathology

After MR scanning, all animals were sacrificed by intraperitoneal injection of 100mg/kg pentobarbital (Dalian Meilun Biotechnology Co., LTD, China). The bilateral kidneys were resected, and tissue samples were sliced using histological microtome (Vicker Science education instrument Co., LTD, China), fixed with 4% formalin (Beijing Lanjieke Technology Co., LTD, China), and embedded in paraffin for hematoxylin and eosin (HE) and periodic acid Schiff (PAS) staining. The sections were examined with light microscopy (NIKON Eclipse ci, Japan) and the images of histopathology were obtained with magnification of 400 ×.

Statistical Analysis

Statistical analysis was performed by using SPSS17.0 statistical software (SPSS, IBM Corp., Armonk, NY). P values < 0.05 were considered to be indicative of significant differences. All quantitative parameters were tested by normal distribution and homogeneity tests of variance. The mean values of laboratory parameters, ADC, and FA of the two groups were calculated and analyzed by the independent two-sample t-test.

RESULTS

Summary of The General Condition and Biochemistry

According to the classification criteria of Mogensen (14), fourteen successful and surviving DN rats met the diagnosis of preclinical diabetic nephropathy.28 kidneys from 14 rats in the DN group and 20 kidneys from 10 rats in the NC group were resected. Streptozotocin-induced diabetes resulted in decreased weight and elevated serum glucose in the DN rats relative to those in the controls ($P = 0.002$ and $P < 0.001$, respectively). The urinary output was more than fourfold higher in the DN rats than in the controls ($P < 0.001$). There was no significant difference in serum creatinine between the two groups ($P > 0.05$). In the DN group, the urinary albumin levels at 2, 4, and 8 weeks were higher than those of the animals in the NC group, but all had values less than 20 ug/min, and there was a statistical difference between the two groups ($P \leq 0.001$; **Table 1**). According to the classification criteria of Mogensen, 14 rats (28 kidneys) met the stage 2 diabetic nephropathy.

Pathology

HE and PAS staining (**Figures 1A, C**, respectively) showed that there were no significant abnormal changes in renal glomeruli and tubules in the NC group. In the DN group, HE staining (**Figure 1B**) showed no obvious abnormality in the structure and morphology of the glomeruli. The structure of renal tubules was unclear or disappeared. The renal tubular epithelial cells were swollen, and the cytoplasm was loose. Some of the tubular epithelial cells were necrotic, and the nucleus showed pyknosis and deep staining or fragmentation. Partial tubular interstitial connective tissue hyperplasia with inflammatory cell infiltration was observed, and a portion of the renal tubular lumen showed necrotic cell fragments. PAS staining (**Figure 1D**) showed that the glomerular basement membrane of the DN rats was slightly thickened, and the PAS-positive area was increased.

MR Imaging

T2-weighted images showed no obvious differences in the morphology and structure of the kidneys between the two groups, and there was clear differentiation between the renal cortex and medulla. Examples of ADC and FA maps in the NC group and DN group are shown in **Figure 2**.

The ADC values of the renal cortex and medulla were significantly higher in the DN rats than in the NC animals ($P < 0.05$; **Table 2**). The FA values of the renal cortex and medulla were significantly lower in the DN group than in the NC group ($P < 0.05$; **Table 3**).

DISCUSSION

Generally, DN is categorized into five stages on the basis of the Mogensen (14) criteria: hyperfiltration stage (stage 1), normal albuminuria stage (stage 2), microalbuminuria stage (stage 3), clinical DN stage (stage 4), and end-stage renal failure (stage 5). Among them, stages 1 and 2 comprise the preclinical stage. Three days after modeling, all animals in the DN group developed hyperglycemia, polyuria, and emaciation. The renal pathological damage in the DN rats, such as tubule

TABLE 1 | General condition and biochemistry ($\bar{x} \pm s$).

	NC group	DN group	t/p
weight(g)	528.8 ± 20.5	373.7 ± 45.5	3.923/0.002
serum glucose(mmol/l)	5.98 ± 1.37	27.46 ± 3.14	-14.283/0.000
serum creatinine(umol/l)	72.92 ± 15.47	85.41 ± 24.91	-0.489/0.634
24h urine volume(mL)	11 ± 1.58	43.25 ± 6.78	-12.909/0.000
urinary albumin at 2 week (ug/min)	4.89 ± 0.84	14.43 ± 3.91	-5.293/0.000
urinary albumin at 4 week (ug/min)	6.11 ± 1.63	16.8 ± 5.27	-4.343/0.001
urinary albumin at 8 week (ug/min)	8.2 ± 1.03	15.50 ± 2.85	-6.593/0.000

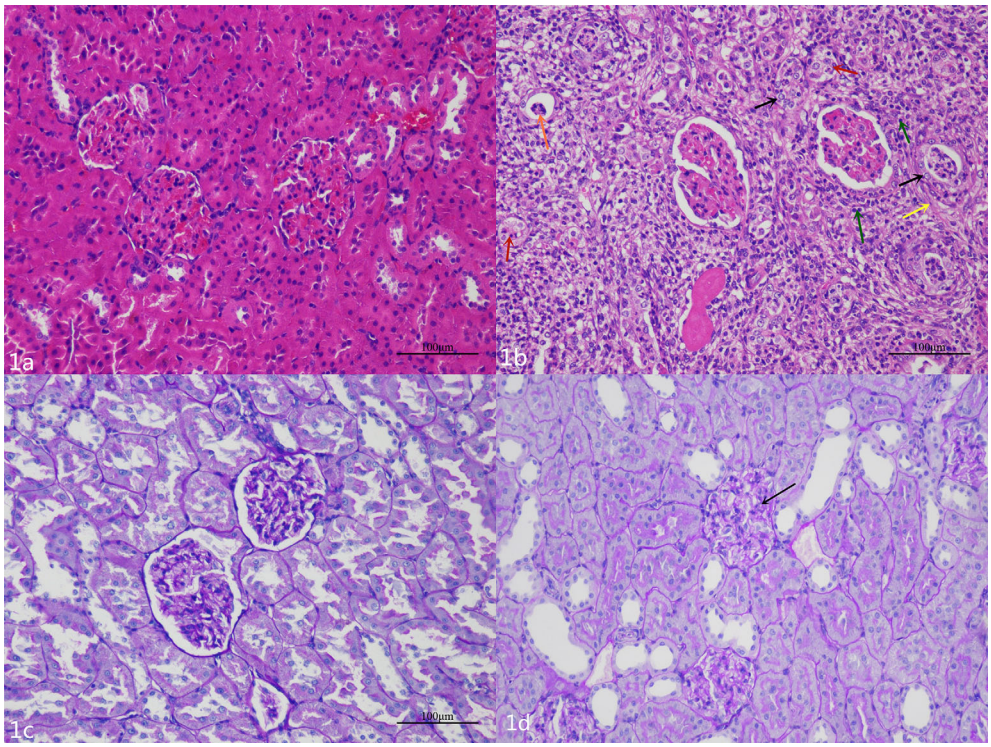

FIGURE 1 | **(A)** The glomeruli and tubules of the NC group are normal. **(B)** Hematoxylin and eosin staining shows that there are different degrees of renal pathological injury in the DN rats. The structure of the renal tissue is disordered, the renal tubular structure was not clear, a large number of renal tubular epithelial cells are swollen, and the cytoplasm is loose (black arrow); a small number of renal tubular epithelial cells are necrotic, and the nucleus is deeply stained or fragmented (red arrows).The renal tubular interstitial connective tissue is hyperplasic (yellow arrow) and accompanied by a small amount of inflammatory cell infiltration (green arrows); necrotic cell fragments can be seen in some renal tubules (orange arrow). **(C)** The renal basement membrane in the NC group is normal. **(D)** The basement membrane is slightly thickened and wrinkled (black arrow), and the mesangial cell is mildly hyperplasic in the DN group.

disappearance, tubule epithelial cell swelling, and inflammatory cell infiltration, were observed, which were in accordance with the preclinical DN stage.

DTI is a promising technique to non-invasively evaluate water molecule diffusion features in the renal parenchyma. Compared with diffusion-weighted imaging, DTI can provide more functional parameters, such as FA, other than ADC. FA is able to provide information about the diffusion direction and its degree at the same time. It has been shown that the FA values of the renal cortex and medulla are reduced in DN patients with or without microalbuminuria relative to those in normal healthy volunteers (6, 15). Yan (5) found that the cortical FA value was significantly lower in early DN rats than in the NC group. The present study showed that the FA values of the renal cortex and medulla were lower in the DN group than in the NC group. These results indicated that the directed diffusion of water molecules in early DN was restricted. The pathological mechanism of FA reduction is not clear. We found that even in the early stage of DN, the kidney showed pathological changes, such as swelling or necrosis of tubular epithelial cells, proliferation of interstitial connective tissue with varying degrees of inflammatory cell infiltration, and filling of cell fragments in the renal tubule lumen, which are in good agreement with those of previous studies (16, 17). Hueper et al. (18) found that reduction of renal FA was significantly and negatively correlated with the extent of renal pathologies, such as glomerulosclerosis, interstitial fibrosis, and tubular damage. Cheung et al. (19) believed that tubular dilation removes part of the directionality of diffusivity along the tubules and therefore explains FA reduction. In addition, at the early stage of DN, the cellular debris congests the tubules, which also weakens the directional diffusion of water molecules (18, 20). We also found that the FA value was lower in the renal cortex than in the medulla in both groups, which was consistent with other study findings (15, 21, 22). This finding may be related to the anatomical characteristics of the kidney. The renal medulla is composed of collecting ducts and some microvascular structures, which arrange radially in the direction of the pelvic cavity. Because of the anatomical characteristics of the renal medulla, the movement of water molecules is more complex in the medulla than in the cortex. But unlike most other studies,our study focuses on preclinical DN, although the urine microalbumin is within the normal range, the renal function has changed at this time. It is possible to provide valuable imaging information for clinical treatment of DN as early as possible.

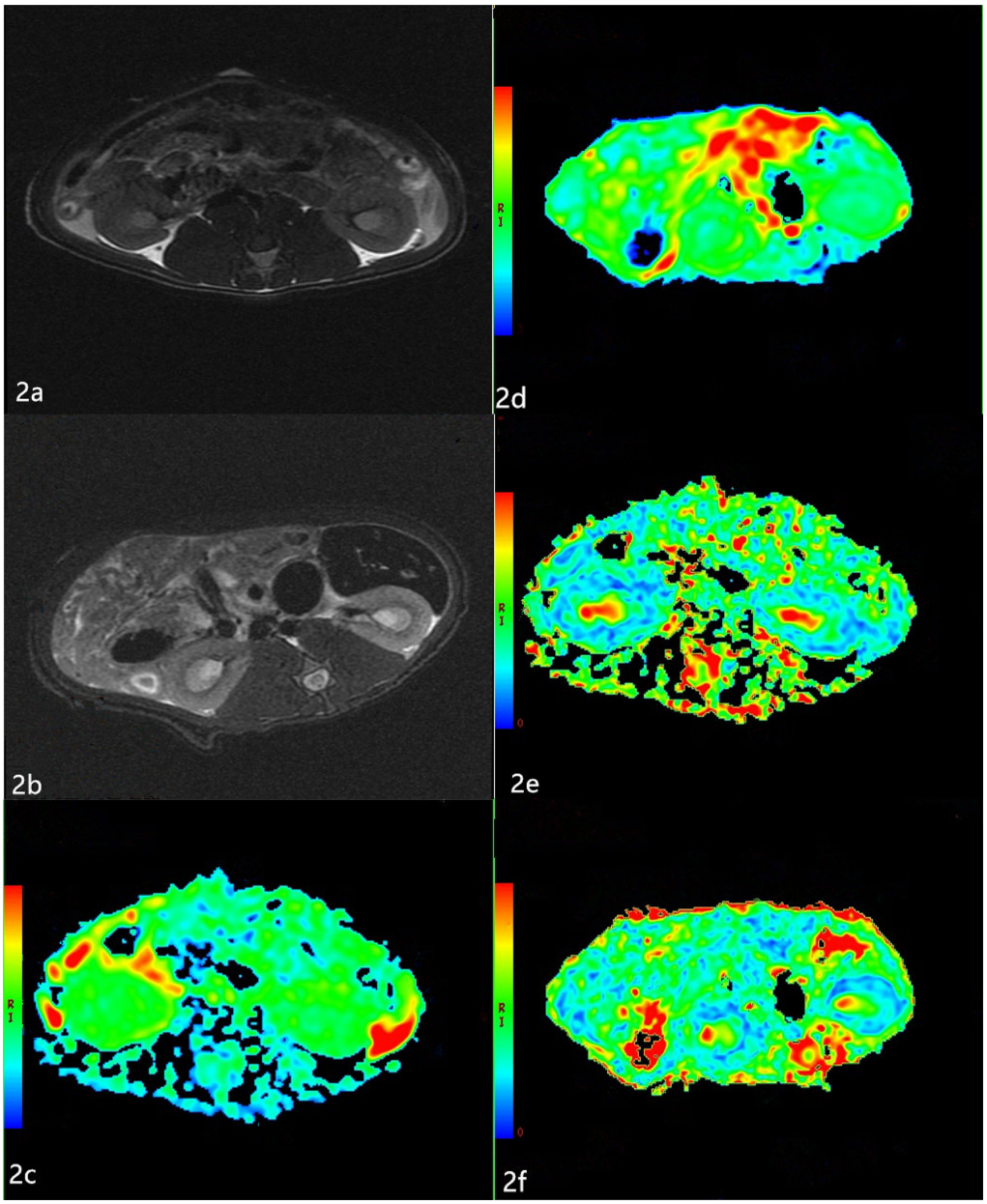

FIGURE 2 | Apparent diffusion coefficients (ADC) and Fractional anisotropy (FA) maps of the two groups **(A, B)** The T2-weighted images of the two groups. T2-weighted images show no obvious changes in the morphology and structure of the kidneys between the two groups, and there is clear differentiation between the renal cortex and medulla. **(C, D)** The ADC maps of the NC group and DN group. The boundary of the cortex and medulla are clear in the NC group. The resolution of the cortex and medulla of the kidneys in the DN group is indistinct. **(E, F)** The FA maps of the NC group and DN group. The boundary of the cortex and medulla are clear, but the color resolution of the renal cortex and medulla is lower in the DN group than in the NC group.

TABLE 2 | The ADC value of renal cortex and medulla ($\times 10^{-3}$ mm^2/s).

Group	Number of kidneys	Cortex	Medulla
NC	20	1.52 ± 0.28 (95%CI 1.37˜1.67)	1.35 ± 0.13 (95%CI 1.28˜1.42)
DN	28	1.75 ± 0.35 (95%CI 1.62˜1.89)	1.53 ± 0.3 (95%CI 1.41˜1.64)
t/p		-2.227/0.028	-2.223/0.032

TABLE 3 | The FA value of renal cortex and medulla.

Group	Number of kidneys	Cortex	Medulla
NC	20	0.26 ± 0.06 (95%CI 0.22˜0.29)	0.30 ± 0.04 (95%CI 0.28˜0.32)
DN	28	0.21 ± 0.05 (95%CI 0.19˜0.23)	0.25 ± 0.06 (95%CI 0.23˜0.27)
t/p		2.345/0.024	3.129/0.003

The diffusion of water molecules is influenced by the water content of the kidney, random motion of water molecules, microcirculation blood flow perfusion, glomerular filtration, reabsorption and secretion in renal tubules, and cell structure (23). In this study, we found that the ADC values of renal cortex and medulla significantly increased in the DN group relative to those in the NC group. These changes may be related to the physiological function of the kidney, which maintains the balance of acid–base and water–salt metabolism mainly through the filtration of glomeruli and reabsorption and secretion in renal tubules. In the preclinical stage of DN, with the increase of renal blood perfusion and the glomerular filtration rate, the amount of water molecules is larger than that in healthy kidneys. However, in preclinical DN, the degree of renal pathological damage is slight, which will not affect the formation and resorption of urine. The diffusion limitation of water molecules caused by renal pathological damage is not obvious at this stage. Therefore, the effect of renal hyperperfusion on the ADC value is greater than that of renal pathological damage, which has been reported by other researchers (5, 24). Cakmak et al. (25) found that the ADC values in patients with stage 3 DN were significantly lower than that in healthy people and they were more obvious in patients with stage 4 and 5 DN. A reduction in the glomerular filtration rate reflects reduction of hyperfiltration. In this way, a lower rate of water transfer across the interstitial space leads to reduced diffusion. When overt proteinuria occurs, histopathological damage is often far advanced. Progressive glomerulosclerosis and tubulointerstitial fibrosis may also restrict water diffusion (26). These effects may oppose the effect of hyperfiltration.

Our study had some limitations. First, only preclinical DN models were included, so we could not compare results with those of stage 3, 4, and 5 DN. Second, this study did not analyze the correlation between DTI parameters and pathological damage. Thirdly, there may be some measurement errors because ROIs were drawn manually, and STZ may has certain nephrotoxicity, which may cause acute kidney injury in rats (27), and interfered with renal changes.

CONCLUSION

In the preclinical DN model, renal cortical and medullary ADC values were significantly increased and FA values were significantly reduced relative to those in healthy animals. DTI might serve a potential role in early, non-invasive, and quantitative diagnosis, and therapy evaluation of DN.

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 animal study was reviewed and approved by the ethics committee of Chengdu First People's Hospital.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Data curation, XH and YW. Formal analysis, YW. Investigation, BP, MK, YY and WL. Methodology, XH, BP, MK, YY and WBL. Project administration, WBL and YW. Writing – original draft, XH. All authors contributed to the article and approved the submitted version.

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Clinical and Pathological Characteristics of Patients With Nonproteinuric Diabetic Nephropathy

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Introduction: As the most common complication of diabetes mellitus (DM), diabetic nephropathy (DN) was initially considered to begin with proteinuria preceding the progression of renal insufficiency. This clinical paradigm has been questioned in the late decades, as many DM patients without proteinuria have progressive renal insufficiency. However, the characteristics of nonproteinuric DN were not fully clear yet.

Patients and Methods: A total of 390 patients with renal biopsy-proven DN in our center were retrospectively recruited in the current study. Clinical and histopathological data of the patients were analyzed. We used propensity score-matching methods to address the imbalance of age, sex, and diabetes duration for comparative analyses.

Results: Among all the renal biopsy-proven DN patients with renal biopsy proven DN, 18 patients were classified as nonproteinuric DN. Compared with 36 propensity score-matched proteinuric DN patients, diabetic retinopathy (DR) was less frequent in nonproteinuric DN patients (38.9% *vs.* 66.4%, $p<0.05$). During the follow-up of 24.0 (12.0–42.0) months, the probability of developing the end-stage renal disease (ESRD) was significantly lower in nonproteinuric DN patients than in proteinuric ones in both the propensity score-matched cohort and overall cohort (log-rank test, $p<0.001$ and $p<0.001$, respectively).

Conclusions: Compared with proteinuric DN patients, DR was less frequent in nonproteinuric DN patients. Nonproteinuric DN patients had better renal outcomes than proteinuric DN patients.

Keywords: diabetic nephropathy, proteinuria, histopathology, outcome, nonproteinuric diabetic nephropathy

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INTRODUCTION

Diabetic nephropathy (DN) is the most common complication of diabetes mellitus (DM) and the leading cause of end-stage renal disease (ESRD) in China (1–3). DN was initially considered to begin with proteinuria preceding the progression of renal insufficiency [estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2]. The natural history was divided into normoalbuminuria (urinary

albumin-to-creatinine ratio [UACR] <30 mg/g), microalbuminuria (UACR 30–300 mg/g), and macroalbuminuria (UACR >300 mg/g), which was mainly based on the typical progression course of type 1 DM (4).

However, this concept of the clinical paradigm has changed over the last decades, and it has been noted that DM patients without proteinuria could also have progressive renal insufficiency. Therefore, the latest diagnostic criteria for diabetic kidney disease (DKD) include low eGFR or the persistent presence of elevated urinary albumin excretion (albuminuria) (5). Nonproteinuric DKD was defined as an eGFR <60 mL/min/1.73 m^2 with a UACR <300 mg/g (6–10). As a diagnosis term, DKD covered both clinical diagnosis and histological diagnosis (DN).

The characteristics of nonproteinuric DN patients are not yet thoroughly investigated. Previous studies showed that the renal histopathological findings of DN are heterogeneous regardless of the level of GFR or UACR (10, 11). According to the previous results, we speculated that nonproteinuric DN patients might have typical histopathological features of DN and a lower risk of CKD progression. Therefore, in the current study, using the cohort of our center and propensity score-matching methods, we investigated clinicopathological characteristics and outcomes in patients with the nonproteinuric phenotype of DN in comparison with patients with the classical proteinuric DN.

PATIENTS AND METHODS

Patients

A total of 390 DM patients with renal biopsy-proven DN who were diagnosed from January 1, 2015, to December 31, 2020, were analyzed retrospectively. DM was defined according to the criteria proposed by the American Diabetes Association in 2017 (12). The investigation was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of Peking University First Hospital (2017-1280). Written informed consent was obtained from each participant.

Among the 390 patients with renal biopsy-proven DN, 298 were male and 92 were female, with an age of 53.11 ± 12.59 years at renal biopsy. The median level of UACR was 2718.56 (1195.57–4897.83) mg/g (**Table 1**). Of the 390 patients, 167 patients who had coexisting non-diabetes-related renal disease, including 54 patients with membranous nephropathy, 45 patients with IgA nephropathy, 15 patients with immune complex-mediated glomerulonephritis, 10 patients with ANCA-associated glomerulonephritis, 7 patients with C3 glomerulonephritis, 6 patients with IgG4-related kidney disease and 30 patients with other renal diseases, were excluded. The comparison between patients with and without coexisting non-diabetes-related renal disease was provided in **Supplementary Table 1**, **Supplementary Figure 1**. 55/390 patients with eGFR>60 ml/min/1.73m^2 were excluded. Ultimately, 168 patients were eligible for further analysis for different proteinuria groups. Among them, 18/168 patients were classified as nonproteinuric DN (UACR <300 mg/g) and 150/168 patients were classified as proteinuric DN (**Figure 1**).

TABLE 1 | Clinical characteristics at the time of renal biopsy (n=390).

Age (years)	53.11 ± 12.59
Male	298 (76.4)
UACR (mg/L)	2718.56 (1195.57-4897.83)
Serum creatinine (μmol/L)	155.55 (104.30-272.72)
eGFR (mL/min/1.73 m^2)	40.29 (20.24-64.24)
≥90	43 (11.0)
60-89	71 (18.2)
45-59	58 (14.9)
30-44	69 (17.7)
15-29	87 (22.3)
<15	62 (15.9)
Diabetes duration (months)	120.0 (60.0-192.0)
Diabetic retinopathy (%)	226 (57.9)
HbA1c (%)	6.7 (6.0-7.8)
Hypertension duration (months)	24.0 (1.0-114.0)

Clinical Characteristics

The clinical data of these patients at the time of renal biopsy and during follow-up were systematically recorded, including age, sex, diabetic retinopathy (DR), use of renin-angiotensin-aldosterone system (RAAS) inhibitors, hemoglobin, serum creatinine (Scr), eGFR, serum albumin, fasting blood glucose (FBG), HbA1c, triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and plasma complements. Proteinuria was expressed as the UACR. Nonproteinuric DN was defined as an eGFR <60 mL/min/1.73 m^2 with a UACR <300 mg/g at the time of renal biopsy according to the previously described criteria (6–10). Cardiovascular disease (CVD) history was self-reported and included a history of congestive heart failure, coronary heart disease, heart attack, angina, stroke, or periphery atherosclerosis. The eGFR was calculated using the CKD-EPI equation (13). HbA1c levels were measured using a high-performance liquid chromatographic assay.

Renal Histopathology

Renal specimens were evaluated using direct immunofluorescence (for immunoglobulins and complement components), light microscopy, and electron microscopy. Periodic acid-Schiff (PAS), silver methenamine, hematoxylin and eosin (HE), and Masson's trichrome staining were used for light microscopy. Biopsies were scored independently by two pathologists. A standard classification system was used based on histological scores for glomerular lesions, tubulointerstitial lesions, vascular lesions and non-diabetic renal lesions (14).

Diabetic glomerulopathy is classified as class I through IV according to the Renal Pathology Society in 2010 (14). Interstitial fibrosis and tubular atrophy (IFTA) were scored semi-quantitatively based on the proportion of the tubulointerstitial compartment affected (0, none; 1, <25%; 2, 25–50%; 3, >50%). Interstitial inflammation was scored semi-quantitatively (0, absent; 1, infiltration only in areas related to IFTA; 2, infiltration in areas without IFTA). Vascular lesions were scored according to the presence of arteriolar hyalinosis and large-vessel arteriosclerosis (grades 0–1) (14). For direct immunofluorescence, the intensities of staining of immunoglobulins, complements, fibrin-associated antigen (FRA), and albumin (Alb) were semi-quantitatively graded on a scale of 0–4+.

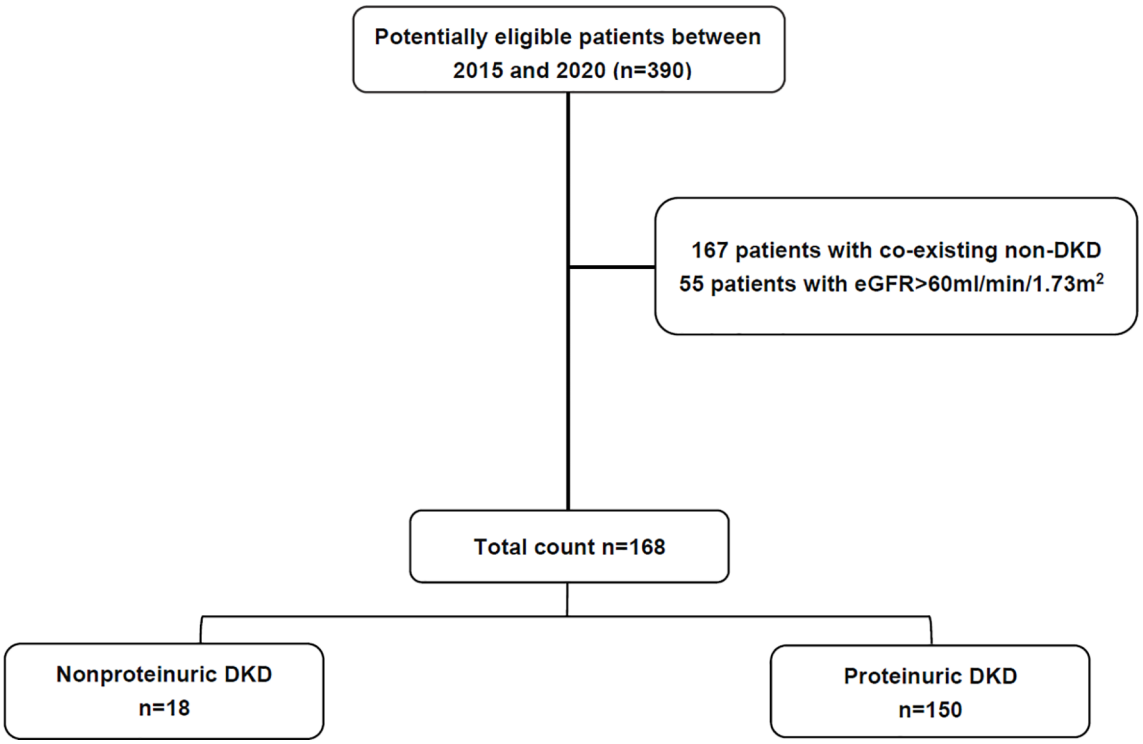

FIGURE 1 | Flowchart for recruitment.

Outcomes

ESRD was defined as the initiation of hemodialysis/peritoneal dialysis, renal transplantation, or death due to uremia. The patients were followed up until the end of 2020 or ESRD, whichever came first. New-onset CVD events included congestive heart failure, coronary heart disease, heart attack, angina, stroke, or periphery atherosclerosis until 2020.

Statistical Analysis

Normally distributed data were presented as mean ± standard deviation, while non-normally distributed data were presented as median values with an inter-quartile range (IQR). Categorical variables were expressed as percentages or ratios. Chi-square, one-way analysis of variance (ANOVA), and t-tests were performed as appropriate. Differences in semi-quantitative and quantitative parameters that were not normally distributed were assessed using Kruskal-Wallis or Mann-Whitney U-tests, as appropriate. Differences were considered significant if the p-value was <0.05. In the current study, the sample size of patients with nonproteinuric DN (n=18) was relatively small compared with the proteinuric DN patients (n=150). We conducted propensity score matching analysis to address the imbalance of background factors such as age, sex, and diabetes duration that affect outcomes. We matched the nonproteinuric DN group with the proteinuric DN group using propensity scores with a one-to-two nearest-neighbor caliper width of 0.01, which is the maximum allowable difference in propensity scores. Analyses were performed using the SPSS statistical software package (version 11.0; Chicago, IL, USA) and R studio 4.0.2.

RESULTS

General Data of the Patients at Renal Biopsy

General data at the renal biopsy of the whole cohort of 390 DN patients were listed in **Table 1**. Among the 18 nonproteinuric DN patients, 13 were male and 5 were female, with 61.39 ± 6.11 years at the time of renal biopsy. The median duration of diabetes was 120.0 (60.0–168.0) months. Seven out of 18 (38.9%) nonproteinuric DN patients complicated with DR. Nine out of 18 (50.0%) patients had hypertension, and the median duration of hypertension was 24.0 (2.0–120.0) months. The median UACR was 147.69 (70.37–279.41) mg/g. The median Scr and eGFR levels were 201.25 (172.00–266.70) μmol/L and 28.81 (21.28–37.46) mL/min/1.73m^2, respectively (**Table 2**).

Comparison of Clinical Manifestations

Clinical features of patients stratified by proteinuria before and after propensity score matching are shown in **Table 2**. Compared with the 36 propensity score-matched proteinuric DN patients, DR was significantly less frequent in nonproteinuric DN patients (38.9% *vs.* 66.4%, p<0.05, respectively). Nonproteinuric DN patients showed a significantly lower level of urinary NAG and a higher level of serum albumin compared with proteinuric DN patients (11.20 [9.00–

TABLE 2 | Clinical features of patients stratified by proteinuria.

	Overall cohort			Propensity score-matched cohort		
	Nonproteinuria DN	Proteinuria DN	P value	Nonproteinuria DN	Proteinuria DN	P value
	n=18	n=150		n=18	n=36	
Age	61.39 ± 6.11	49.80 ± 6.42	<0.001	61.39 ± 6.11	59.86 ± 7.19	0.536
Male/Female	13/5	113/37	0.083	13/5	24/12	0.679
Diabetes duration (months)	120.0 (60.0,168.0)	120.0 (72.0,192.0)	0.621	120.0 (60.0,168.0)	120.0 (84.0,216.0)	0.592
Diabetic retinopathy (%)	38.9	78.7	<0.001	38.9	66.4	0.031
CVD history (%)	44.4	44.7	1	44.4	63.9	0.173
Hypertension duration (months)	24.0 (2.0,120.0)	24.0 (4.0,84.0)	1	24.0 (2.0,120.0)	66.0 (24.0,240.0)	0.119
Fasting blood glucose (mmol/L)	5.84 (5.12,8.90)	6.38 (5.41, 7.80)	0.894	5.84 (5.12,8.90)	6.01 (5.41,7.08)	0.808
HbA1c (%)	6.45 (6.15,7.55)	6.60 (5.90,7.60)	0.712	6.45 (6.15,7.55)	6.40 (6.10,7.70)	0.977
Urine NAG (U/L)	11.20 (9.00, 14.50)	24.00 (13.25,47.00)	0.001	11.20 (9.00, 14.50)	23.80 (13.70,54.00)	0.002
Urine α1-microglobulin (mg/L)	51.40 (27.2,79.70)	68.10 (39.75,109.00)	0.302	51.40 (27.2,79.70)	73.65 (46.20,127.50)	0.181
Hemoglobin (g/L)	109.78 ± 20.52	104.69 ± 19.24	0.467	109.78 ± 20.52	105.50 ± 18.89	0.449
Scr (μmol/L)	201.25 (172.00,266.70)	227.92 (153.01,351.50)	0.538	201.25 (172.00,266.70)	228.30 (169.93, 349.28)	0.419
eGFR (mL/min/1.73 m^2)	28.81 (21.28,37.46)	25.97 (15.28,41.60)	0.922	28.81 (21.28,37.46)	25.85 (13.37,33.08)	0.497
Serum albumin (g/L)	41.11 ± 3.61	31.70 ± 5.49	<0.001	41.11 ± 3.61	32.65 ± 5.81	<0.001
Platelet (×10^9/L)	209.65 ± 73.64	224.39 ± 76.95	0.088	209.65 ± 73.64	190.90 ± 75.15	0.41
Uric acid (μmol/L)	365.22 ± 106.47	427.63 ± 116.81	0.032	365.22 ± 106.47	429.11 ± 146.73	0.107
LDL-cholesterol (mmol/L)	2.07 (1.71,2.37)	2.85 (2.07,3.56)	0.001	2.07 (1.71,2.37)	2.80 (2.10,3.42)	0.008
HDL-cholesterol (mmol/L)	0.81 (0.64,0.99)	0.93 (0.80,1.14)	0.011	0.81 (0.64,0.99)	0.92 (0.84,1.12)	0.026
Triglyceride (mmol/L)	2.00 (1.51, 2.92)	1.89 (1.28,2.90)	0.61	2.00 (1.51, 2.92)	1.83 (1.28,2.52)	0.428
Serum C3	0.94 (0.78,1.12)	0.87 (0.75,0.99)	0.303	0.94 (0.78,1.12)	0.86 (0.74,1.04)	0.266
Serum C4	0.27 (0.20,0.33)	0.27 (0.22,0.33)	0.687	0.27 (0.20,0.33)	0.23 (0.19,0.32)	0.443
RAAS inhibitor	4 (22.2%)	42 (28.0%)	0.76	4 (22.2%)	11 (30.6%)	0.519

Chi-square tests were performed in percentages or ratios variables. T-tests were performed in normally distributed variables. semi-quantitative and quantitative parameters that were not normally distributed were assessed using Kruskal-Wallis or Mann-Whitney U-tests.

Values are expressed as a mean ± standard deviation, percentage or median with upper and lower quartile or percentage.

14.50] U/L *vs.* 23.80 [13.70–54.00] U/L, p<0.05; 41.11 ± 3.61 g/L *vs.* 32.65 ± 5.81 g/L, p<0.001, respectively). Significantly lower LDL-cholesterol and HDL-cholesterol levels were observed in nonproteinuric DN patients compared with proteinuric DN patients [2.07 (1.71–2.37) mmol/L *vs.* 2.80 (2.10–3.42) mmol/L, p<0.05; 0.81 (0.64–0.99) mmol/L *vs.* 0.92 (0.84–1.12) mmol/L, p<0.05, respectively]. There was no significant difference in RAAS inhibitor use between the two groups.

Comparison of Renal Histopathological Features

Detailed renal histopathological manifestations are shown in **Table 3**. According to the international consensus classification of DN proposed in 2010, most nonproteinuric DN patients showed typical diabetic glomerulopathy, including mesangial expansion or nodular sclerosis (Kimmelstiel-Wilson lesions), 3 (16.7%), 11 (61.1%), 3 (16.7%), and 1 (5.5%) of whom were categorized as class I, class II, class III, and class IV, respectively. Varying degrees of tubulointerstitial damage were found in nonproteinuric DN patients.

Compared with proteinuric DN patients, nonproteinuric DN patients had milder glomerular injuries (**Table 3**). For example, advanced DN pathology manifestations (class III and class IV) were observed in only 4/18(22.2%) of nonproteinuric DN patients, whereas they were found in 27/36(75.0%) of matched proteinuric ones. No significant difference in tubulointerstitial damage was found between the two matched groups. The proportion of patients with arteriolar hyalinosis was significantly lower in the nonproteinuric DN group than in matched proteinuric group (66.7% *vs.* 88.9%, p<0.05). All nonproteinuric and proteinuric DN patients showed arteriosclerosis in the kidneys (**Table 3**).

Regarding direct immunofluorescence, there were significantly lower proportions of IgM and C1q depositions in nonproteinuric DN patients than in matched proteinuric ones (11.1% *vs.* 77.8%, p<0.001 and 0.0% *vs.* 58.3%, p<0.05, respectively) (**Table 3**). A significantly higher proportion of C3 deposition was found in patients with proteinuria in the overall cohort (44.4% *vs.* 72.0%, p<0.05) (**Table 3**).

Outcomes

During a median follow-up duration of 24.0 (12.0–42.0) months, none of the nonproteinuric DN patients progressed to ESRD, whereas 21/36 (65.6%) of the matched proteinuric DN patients progressed to ESRD. Among the patients with proteinuria from the overall cohort, 92/150 (61.3%) progressed to ESRD. Kaplan-Meier analysis showed that the probability of developing ESRD was significantly lower in nonproteinuric DN patients than in proteinuric ones in both the propensity score-matched cohort and overall cohort (log-rank test, p<0.001 and p<0.001, respectively) (**Figure 2**). Only 1/18 patients with nonproteinuric DN and 22/150 patients with proteinuria DN had new-onset CVD in the current study (P>0.05), which might be due to the relatively short follow-up.

DISCUSSION

DN is the leading cause of ESRD and is associated with increased cardiovascular morbidity and all-cause mortality (15–17). Traditionally, persistent microalbuminuria has been considered

TABLE 3 | Renal histopathological features of patients stratified by proteinuria.

	Overall cohort			Propensity score-matched cohort		
	Nonproteinuric DN n=18	**Proteinuria DN n=150**	**P value**	**Nonproteinuric DN n=18**	**Proteinuria DN n=36**	**P value**
Glomerular classification			<0.001			0.001
Class I/Class II/Class III/Class IV	3/11/3/1	1/25/99/25		3/11/3/1	0/9/19/8	
Interstitial lesions						
IFTA			0.074			0.107
0/1/2/3	0/4/11/3	0/17/68/65		0/4/11/3	0/9/12/15	
Interstitial inflammation			0.143			0.319
0/1/2	0/5/13	0/36/114		0/5/13	0/15/21	
Vascular lesions						
Arteriolar hyalinosis			0.034			0.048
0	6	19		6	4	
1	12	131		12	32	
Arteriosclerosis			NA			NA
0	0	0		0	0	
1	18	150		18	36	
IgG deposition (0/≥1)	12/6	28/122	0.143	12/6	10/26	0.673
IgM deposition (0/≥1)	16/2	47/103	<0.001	16/2	8/28	<0.001
IgA deposition (0/≥1)	14/4	41/109	0.644	14/4	9/27	0.822
C3 deposition (0/≥1)	10/8	42/108	0.017	10/8	11/25	0.076
C1q deposition (0/≥1)	18/0	45/105	0.007	18/0	15/21	0.001
Alb deposition (0/≥1)	13/5	28/122	0.358	13/5	9/27	0.826

Values are expressed as a mean ± standard deviation, percentage or median with upper and lower quartile or percentage.
Chi-square tests were performed in percentages or ratios variables.

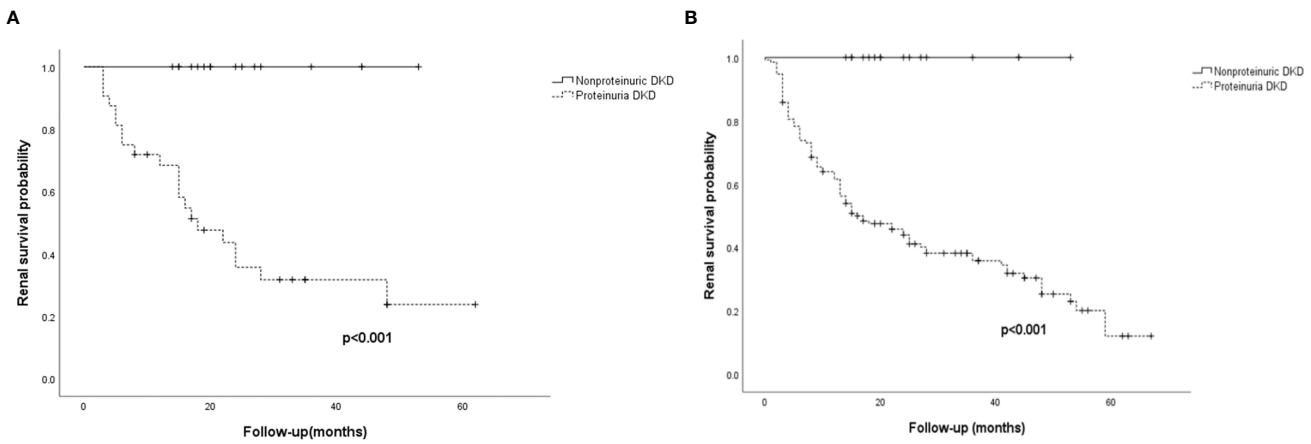

FIGURE 2 | Renal survival for the 54 patients in the propensity score-matched cohort and the 168 patients in the overall cohort. **(A)** Kaplan-Meier curves of renal survival in the propensity score-matched cohort. **(B)** Kaplan-Meier curves of renal survival in the overall cohort. ESRD was defined as initiation of hemodialysis/peritoneal dialysis, renal transplantation, or death as a result of uremia. Nonproteinuric DN was defined as patients with an eGFR<60 mL/min/1.73 m^2 without proteinuria (UACR<300 mg/g); proteinuria DN was defined as patients with an eGFR<60 mL/min/1.73 m^2 and proteinuria (UACR>300 mg/g).

the first clinical sign of DN, inevitably progressing to macroalbuminuria and subsequent renal dysfunction (18). However, over recent decades, there has been increasing recognition that GFR reduction may precede the development of proteinuria in several patients with diabetes (6–8, 19, 20). These patients were therefore defined as nonproteinuric DKD/DN. The prevalence of proteinuric DKD declined, while the prevalence of nonproteinuric DKD increased, attributable to a higher rate of RAAS inhibitors prescription (21). Although the paradigm has been renewed, the characteristics of nonproteinuric DN have not been thoroughly investigated.

In patients with DKD, the prevalence of nonproteinuria varies between 20% and 40% (22, 23). In the current study, a total of 18/223 (8.1%) DN patients were classified as nonproteinuric DN, which was lower than that in previous reports. Of the patients with reduced eGFR (<60 mL/min/1.73 m^2) from the National

Health and Nutrition Examination Survey (NAHNES III) in 2003, 81% had nonproteinuric DKD, and only 19% had proteinuria (19). In the UK Prospective Diabetes Study (UKPDS-74), during 15 years of follow-up in 4,006 patients with type 2 diabetes, 1,132 (28.3%) developed renal impairment. Of the latter, 575 (50.8%) patients were classified as nonproteinuric DKD (24). We have noted that all patients in the current study underwent renal biopsy, which was not highly recommended in nonproteinuric DKD patients unless they were suspected of having either superimposed non-diabetic kidney disease or *de novo* non-diabetic kidney disease (25). The relatively lower prevalence of nonproteinuric DN patients in the current study might be associated with the lower rate of renal biopsy in this subgroup of patients. In summary, the prevalence of nonproteinuric DKD is not low. The traditional nonproteinuric DKD should also be paid attention and concern on, mainly due to lower eGFR and renal insufficiency.

Compared with proteinuric DN patients, a significantly lower proportion of DR in nonproteinuric DN patients was found in both the overall and the matched cohorts. The prevalence of DR in patients with nonproteinuric DKD varies across studies. A study from RIACE with 2,959 DKD patients found that 2,028 (68.5%) patients did not have DR, and 538 patients (18.2%) showed both proteinuria and retinopathy (26). The varying prevalence of DR suggests that the development of nonproteinuric DKD may be independent of diabetic microangiopathic lesions (19, 23).

Only a limited number of studies have investigated the renal histopathological features of nonproteinuric DN. Results from previous biopsy-based studies were inconsistent, which may be due to the small sample size and the timing of renal biopsy. Studies of the renal histopathology in patients with type 2 DM showed that nonproteinuric patients had less frequent typical glomerular injuries. The findings were not consistent for tubulointerstitial and arterial injuries (11, 27, 28). Yamanouchi et al. reported that patients with nonproteinuric DN have both milder glomerular injuries and tubulointerstitial injuries (10). In the current study, consistent with previous reports, most of the nonproteinuric DN patients showed typical but milder glomerular injuries, including mesangial expansion and nodular sclerosis (Kimmelstiel-Wilson lesions), while tubulointerstitial injuries were heterogeneous. More importantly, these results suggest that typical glomerular injuries may precede overt proteinuria in DN. For immunofluorescence, there was a significantly lower proportion of IgM and C1q deposition in nonproteinuric DN patients compared with matched proteinuric DN patients. A higher proportion of C3 deposition was found in patients with proteinuria in the overall cohort. Previous studies have shown that complement deposition in renal histopathology is associated with severe kidney damage in DN patients (29, 30). Persistent proteinuria may induce local complement activation and aggravate renal injury. The pathogenic role of complement overactivation warrants further investigation.

In the current study, the renal outcome was more favorable in nonproteinuric DN patients than those with proteinuria. None of the nonproteinuric DN patients progressed to ESRD. These results were consistent with previous studies (31, 32). Proteinuria remains a crucial independent predictor of eGFR decline in DM patients, especially those with low eGFR. However, even if the risk for ESRD was low, nonproteinuric patients showed an equal or even higher risk of CVD morbidity and mortality than those with proteinuria (33–37). The results suggest that nonproteinuric DN may represent a distinct phenotype, with macroangiopathic and tubulointerstitial lesions instead of microangiopathic lesions involved in the underlying pathology. Close attention and care for CVD morbidity and mortality in these patients are needed.

This study has some limitations. First, the sample size was small, and the follow-up duration was short for assessing the probability of developing ESRD. The current study was a single-center study that recruited only 18 nonproteinuric DN patients. Therefore, the true prevalence of nonproteinuric DKD cannot be accurately assessed. Second, there was an inevitable bias in patients receiving renal biopsy. Third, we only referred to Chinese DN patients in the current study. Studies involving multi-ethnic and multi-center are needed.

CONCLUSION

In conclusion, compared with proteinuric DN patients, DR was less frequent in nonproteinuric DN patients. Nonproteinuric DN patients had better renal outcomes than proteinuric patients. Multicenter studies with larger sample sizes are needed to further understand nonproteinuric DN.

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 Ethics Committee of Peking University First Hospital (2017-1280). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

D-YC and MC designed the study. D-YC, M-RL, X-JY, and S-XW contributed data. D-YC and MC drafted the analysis plan. D-YC performed the statistical analysis. D-YC wrote the manuscript. MC and M-HZ revised the manuscript and supervised the study. MC is the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of

the 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.2021.761386/full#supplementary-material

Supplementary Table 1 | Clinical features of DN patients with non-diabetic renal disease (NDRD) and without NDRD. Values are expressed as a mean ± standard deviation, percentage or median with upper and lower quartile or percentage. Chi-square tests were performed in percentages or ratios variables. T-tests were performed in normally distributed variables. semi-quantitative and quantitative parameters that were not normally distributed were assessed using Kruskal-Wallis or Mann-Whitney U-tests.

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Association of Urinary Sodium Excretion and Diabetic Kidney Disease in Patients With Type 2 Diabetes Mellitus: A Cross-Sectional Study

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Background: Diabetic kidney disease (DKD) is the leading cause of end-stage kidney disease worldwide. Epidemiological evidence of the association between urinary sodium excretion and the presence of DKD in patients with type 2 diabetes mellitus (T2DM) has not yet been well established.

Methods: We performed a cross-sectional study of 1545 patients with T2DM over aged 20 years old from January 2018 to December 2020. Urinary sodium excretion was measured by 24-hour urine samples in inpatients and morning fasting urine samples in outpatients. The associations between urinary sodium excretion and the risks of DKD were examined using stepwise regression analysis, logistic regression analysis and multivariable-adjusted restricted cubic splines (RCS).

Results: Regression analysis showed that urinary sodium was independently associated with urinary albumin to creatinine ratio (UACR) level (P = 0.006) and the risks of DKD (P = 0.042). In multivariable-adjusted RCS analysis, urinary sodium excretion was significantly associated with UACR in all patients (P = 0.008), and exhibited a J-shaped relationship. Logistic regression analysis showed that increased urinary sodium excretion was significantly associated with increased risks of DKD [OR (95% CI); 1.56 (1.07-2.27); P = 0.020]. However, the relationships between urinary sodium excretion and the risks of DKD and albuminuria showed no significance, after further adjustment for HOMA-IR and ba-PWV (brachial-ankle pulse wave velocity) (Both P > 0.05).

Conclusions: Higher urinary sodium excretion level was associated with increased risks of DKD among patients with T2DM, dependent of vascular sclerosis and insulin resistance.

Keywords: diabetic kidney disease, type 2 diabetes, urinary sodium excretion, insulin resistance, vascular sclerosis

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INTRODUCTION

Diabetic kidney disease (DKD), one of the common complications of diabetes mellitus, was strongly associated with all-cause and cardiovascular disease (CVD) mortality in a multiethnic Asian population (1). DKD is the leading cause of end-stage kidney disease worldwide, accounting for approximately 50% of cases in the developed countries (2). The World Health Organization (WHO) multinational study showed that renal disease including DKD accounted for 11% of all deaths of patients with type 2 diabetes mellitus (T2DM) (3). In China, approximately 24.3 million have DKD and 60.5% of patients with diabetes have reduced kidney function or slightly increased albuminuria (4).

It has been well documented that sodium intake is positively associated with the risks of clinical CVD events and chronic kidney disease (CKD) (5, 6). However, the association between sodium intake and the risks of DKD remains less clear. Albuminuria has emerged as a sensitive marker of kidney damage (7), and a predictive risk factor for end-stage renal failure in diabetic individuals (8). Epidemiological evidence conducted in the general population and patients with type 1 diabetes and reported that sodium intake was positively related to urinary albumin excretion (9, 10), particularly in overweight subjects (10). Additionally, few studies reported a reverse J-shaped or no significant association between dietary sodium and urinary albumin in patients with T2DM (11, 12). Indeed, dietary sodium intake measurement methods may contribute to these conflicting findings. However, these evidences did not provide a conclusive information regarding the association between sodium intake and DKD in patients with T2DM. We quantified urinary sodium excretion by 24-hour urine samples, which be considered the most reliable estimate of sodium intake (13), and aimed to explore the associations between urinary sodium excretion and the risks of DKD in patients with T2DM.

MATERIALS AND METHODS

Study Participants

This cross-sectional study was based on the Nanfang Prospective Diabetes Study (NFPDS), a prospective cohort study designed to explore the associations of urinary electrolyte and possible risk factors of microvascular complications in patients with T2DM from Nanfang Hospital, Southern Medical University. We followed the methods of Liu et al. (14). A total of 1545 inpatients and outpatients over aged 20 years old from Guangzhou city, China, was included in this study from January 2018 to December 2020. In the present study, participants completed urine collections and evaluation of diabetic microvascular complications. Inclusion criteria was diagnosis of T2DM according to the criteria of the American Diabetes Association (ADA) (2017) (15). The individuals undergoing dialysis treatment, being pregnant or planning to become pregnant, and patients with NYHA class III or IV congestive heart failure and severe systemic infection were excluded. All participants completed a uniform questionnaire regarding demographics, lifestyle habits (i.e., smoking status, alcohol consumption) and medical history.

The protocol for the study received ethical approval conforming to the Declaration of Helsinki from the Institutional Review Board of Nanfang Hospital, Southern Medical University. All participants provided written informed consent.

Measurements

Physical examination included height, weight and blood pressure (BP) were screened following a standardized protocol. The height and weight were measured by using the same automatic measuring instrument. Body mass index (BMI) was calculated as weight (kg) divided by square of height (m^2). BP was measured in triplicate with an electronic sphygmomanometer (OMRON Company). The mean value of the three readings were used for analysis. ba-PWV (brachial-ankle pulse wave velocity, ba-PWV) was measured using an arteriosclerosis detection device (Omron, BP-203RPEIII).

Overnight fasting blood samples of inpatients and outpatients were obtained and tested in the laboratory of Nanfang hospital with stringent quality control. Triglyceride (TG), total Cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), creatinine (CR), uric acid (UA) was determined by enzymatic methods using a fully automated biochemical analyzer. Urinary creatinine (Ucr), urinary albumin to creatinine ratio (UACR) were determined by automatic protein - specific analyzer (Afinion AS100). Glycated hemoglobin (HbA1c) was determined by high performance liquid chromatography. Fasting plasma glucose concentrations were determined by the hexokinase method. Fasting insulin level was determined by electroluminescent immunoassay. According to the test results, index of homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the following formula: HOMA-IR = fasting insulin (mIU/L) ×FBG (mmol/L)/ 22.5. Inpatients were asked to collect 24-hour urine samples for the measurement of urinary sodium level. Outpatients were asked to collect morning fasting urine samples to measure spot urinary sodium. The Kawasaki formula was used to estimate 24-hour urinary sodium excretion of outpatients, which be considered valid for estimating sodium intake in healthy participants and patients with antihypertensive therapy (16, 17). 24-hour and spot urinary sodium concentrations were measured by ion selective electrode method.

On the basis of the mean of three seated, hypertension was defined as mean BP of 140/90 mmHg or greater and/or the self-reported use of antihypertensive medication. Hyperlipidemia was defined as total cholesterol (TC) ≥ 6.22mmol/l, or low-density lipoprotein cholesterol (LDL-c) ≥ 4.14 mmol/l, or triglycerides (TG) ≥ 2.26 mmol/l and/or self-reported use of lipid-lowering drugs.

Definitions of Albuminuria and DKD

Albuminuria was diagnosed as UACR ≥ 30 mg/g, while excluding infection and other factors. The value of UACR was obtained by calculating urinary albumin (mg) to creatinine (g) ratio. DKD was diagnosed as UACR ≥ 30 mg/g and/or eGFR < 60

$ml \cdot min^{-1} \cdot (1.73\ m^2)^{-1}$, while excluding other causes of chronic kidney disease. The estimated glomerular filtration rate (eGFR) was calculated by using the CKD-EPI formula (18). Definitions as described above were obtained according to the criteria of ADA (2017) (19).

Statistical Analysis

Baseline characteristics were described as means ± standard deviation (SD), median (interquartile range), frequencies, or percentage. Data that were not normally distributed were logarithmically transformed before analysis. General linear models (GLM) and the chi-square test were used to compare the differences between the four quartiles of urinary sodium excretion. The stepwise regression was used to determine the relationship between variables and DKD. Multivariate logistics regression model was used to estimate relationship between urinary sodium excretion level and the risk of DKD as well as albuminuria. RCS model was used to investigate the relationship between urinary sodium excretion and UACR level. Forest plot was used to examine the relationship between urinary sodium excretion levels and the risk of DKD in different subgroups. According to the recommendations of WHO (20), urinary sodium excretion of less than 2 g/d was selected as the reference group for all spline plots. Statistical analyses were performed by using SAS version 9.4 (SAS Institute Inc). A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 presents the clinical characteristics of patients categorized by presence of DKD. The mean age of the subjects with DKD was 56.5 ± 10.6 years. Subjects with DKD had higher urinary sodium excretion level than those with non-DKD (3.42 ± 1.48 g/d *vs.* 3.23 ± 1.42 g/d, respectively, $P = 0.029$). Subjects with DKD exhibited greater age, longer duration of diabetes, higher levels of SBP, DBP, PWV, TG, CR, UA, UACR than those with non-DKD (All $P < 0.05$). Likewise, The DKD group had higher prevalence of hyperlipidemia and hypertension than the non-DKD group. Furthermore, patients with DKD had higher percentage of using RAS blocking agents, diuretics, statin, SGLT 2i (All $P < 0.05$). The level of eGFR was lower in patients with DKD compared to those with non-DKD ($P < 0.001$). There were no significant differences in the levels of BMI, HbA1c, glucose, HOMA-IR, TC and LDL-c between two groups (All $P > 0.05$).

The baseline characteristics of participants categorized by quartile of urinary sodium excretion are showed in **Table 2**. The median of duration of diabetes was 7 (2-12) years. Patients in the higher quartiles had higher prevalence of smoking, longer duration of diabetes and higher percentage of using antidiabetic medication than those in the lower quartiles (All $P < 0.05$). In addition, there was a significant increasing trend in the levels of BMI, HOMA-IR and eGFR with increasing urinary sodium excretion. Conversely, there was a significant decreasing trend in the levels of HbA1c, TC and LDL-c with increasing urinary sodium excretion. Levels of SBP, DBP, PWV, TG, UR, UA and UACR, glucose showed no differences among the four quartiles of urinary sodium excretion (All $P > 0.05$). Of note, the prevalence of DKD from the lowest quartile to the highest quartile was 19.7%, 20.0%, 21.0%, 26.4%, respectively ($P = 0.028$), adjusted for age and gender.

TABLE 1 | Characteristics of patients categorized by presence of DKD.

Variables	non-DKD	DKD	*P*-value
Sample size	1209	336	–
Urinary sodium (g/d)	3.23 ± 1.42	3.42 ± 1.48	0.029
Age (years)	54.5 ± 11.0	56.5 ± 10.6	0.004
Gender (Male n, %)	772 (63.9)	219 (65.2)	0.654
Smoking (n, %)	545 (45.1)	167 (50.2)	0.103
Alcohol use (n, %)	398 (33.0)	126 (37.8)	0.095
Duration of diabetes (years)	6 (2-11)	11 (5-16)	<0.001
BMI (kg/m^2)	24.5 ± 3.5	24.7 ± 3.8	0.290
SBP (mmHg)	125.8 ± 17.5	133.4 ± 19.4	<0.001
DBP (mmHg)	76.7 ± 11.0	79.2 ± 10.7	<0.001
Hypertension (n, %)	353 (29.2)	184 (54.8)	<0.001
Hyperlipidemia (n, %)	652 (53.9)	246 (73.2)	<0.001
Antidiabetic medication (n, %)	847 (70.1)	284 (84.5)	<0.001
RAS blocking agents (n, %)	133 (11.0)	107 (31.9)	<0.001
Diuretics (n, %)	31 (2.6)	17 (5.1)	0.020
Statin (n, %)	132 (10.9)	76 (22.6)	<0.001
SGLT 2i (n, %)	19 (1.6)	13 (3.9)	0.009
PWV (cm/s)	1571.6 ± 311.9	1736.4 ± 346.9	<0.001
HbA1c (%)	9.3 ± 2.6	9.4 ± 2.4	0.579
Glucose (mmol/L)	8.7 ± 4.4	8.7 ± 5.1	0.876
HOMA-IR	0.70 ± 1.01	0.73 ± 1.18	0.667
TG (mmol/l)	1.44 (0.98-2.26)	1.65 (1.08-3.05)	0.003
TC (mmol/l)	5.00 ± 1.30	5.07 ± 1.58	0.381
LDL-c (mmol/l)	3.21 ± 0.94	3.23 ± 0.97	0.712
CR (μmol/l)	66.0 (55.0-78.0)	80.5 (60.0-111.0)	<0.001
UA (μmol/l)	354.16 ± 110.10	377.63 ± 125.79	<0.001
UACR (mg/mmol)	1.1 (0.7-2.6)	9.4 (3.3-46.0)	<0.001
eGFR ($ml/min/1.73m^2$)	95.83 ± 22.47	79.52 ± 31.97	<0.001

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; RAS, renin-angiotensin system; PWV, pulse wave velocity; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; TG, triglyceride; TC, total cholesterol; LDL-c, low-density lipoprotein cholesterol; CR, creatinine; UA, uric acid; UACR, urinary albumin to creatinine ratio; eGFR, estimated glomerular filtration rat.

As shown in **Table 3**, the levels of PWV, HbA1c, CR, UA and urinary sodium were independently correlated with UACR level and the presence of DKD in stepwise regression analysis (All $P < 0.05$). In addition, age, gender, and the levels of BMI, DBP, SBP, TC were significantly correlated with UACR level.

The RCS analysis showed that urinary sodium excretion was significantly associated with UACR level in all patients ($P = 0.008$) and males ($P = 0.017$), after adjusted for age, gender, BMI, smoking, alcohol consumption, DBP, HbA1c, use of RAS blocking agents, diuretics, hyperlipidemia, statin, and antidiabetic drugs (**Figure 1**). However, there was insignificant nonlinear association between urinary sodium excretion and UACR level ($P = 0.081$). A J-shaped relationship was observed between urinary sodium excretion and UACR level.

The multivariable-adjusted odds ratios (ORs) for the association between urinary sodium excretion and the risks of DKD and albuminuria are shown in **Table 4**. After adjustment for age, gender, BMI, smoking, alcohol consumption, DBP, HbA1c, and use of RAS blocking agents, diuretics, hyperlipidemia, statin and antidiabetic medication, individuals

TABLE 2 | Characteristics of patients categorized by quartile of urinary sodium excretion level.

Variables	Total	Estimated 24-hour urinary sodium excretion level				*P*-value
		Quartile 1	Quartile 2	Quartile 3	Quartile 4	
Sample size	1545	386	386	386	387	–
Urinary sodium (g/d)	3.27 ± 1.44	1.69 ± 0.45	2.72 ± 0.25[b]	3.51 ± 0.25[b]	5.16 ± 1.21[b]	<0.001
Age (years)	54.9 ± 10.9	54.8 ± 11.6	55.5 ± 10.7	55.4 ± 10.7	54.0 ± 10.6	0.226
Gender (Male n, %)	991 (64.1)	218 (56.5)	236 (61.1)	257 (66.6)	280 (72.4)	<0.001
Smoking (n, %)	712 (46.2)	161 (41.7)	166 (43.1)	188 (49.0)	197 (51.0)	0.024
Alcohol use (n, %)	524 (34.0)	116 (30.1)	127 (33.0)	144 (37.5)	137 (35.5)	0.149
Duration of diabetes (years)	7 (2-12)	4 (2-10)	7 (2-12) [b]	8 (2-13)[b]	7 (2-13) [b]	<0.001
BMI (kg/m^2)	24.5 ± 3.5	23.9 ± 3.5	24.4 ± 3.5[a]	24.5 ± 3.1[a]	25.4 ± 3.7[b]	<0.001
SBP (mmHg)	127.4 ± 18.3	126.4 ± 18.4	126.6 ± 18.6	127.9 ± 18.6	128.9 ± 17.4	0.174
DBP (mmHg)	77.2 ± 11.0	77.0 ± 11.7	76.9 ± 10.5	76.7 ± 10.7	78.4 ± 10.9	0.127
Hypertension (n, %)	537 (34.8)	152 (39.4)	131 (33.9)	123 (31.9)	131 (33.9)	0.150
Hyperlipidemia (n, %)	898 (58.1)	227 (58.8)	226 (58.6)	228 (59.1)	217 (56.1)	0.822
Antidiabetic medication (n, %)	1131 (73.2)	238 (61.7)	283 (73.3)	306 (79.3)	304 (78.6)	<0.001
RAS blocking agents (n, %)	240 (15.5)	61 (15.8)	56 (14.5)	67 (17.4)	56 (14.5)	0.650
Diuretics (n, %)	48 (3.1)	18 (4.7)	10 (2.6)	9 (2.3)	11 (2.8)	0.230
Statin (n, %)	208 (13.5)	46 (11.9)	52 (13.5)	57 (14.8)	53 (13.7)	0.712
SGLT 2i (n, %)	32 (2.1)	4 (1.0)	10 (2.6)	7 (1.8)	11 (2.8)	0.282
PWV (cm/s)	1607.1 ± 326.8	1602.0 ± 333.3	1602.7 ± 335.6	1602.7 ± 319.9	1621.3 ± 319.0	0.825
HbA1c (%)	9.4 ± 2.5	10.1 ± 2.8	9.5 ± 2.5[b]	8.9 ± 2.4[b]	8.9 ± 2.2[b]	<0.001
Glucose (mmol/L)	8.7 ± 4.6	8.9 ± 5.4	8.8 ± 4.5	8.3 ± 3.9	8.6 ± 4.3	0.278
HOMA-IR	0.71 ± 1.04	0.46 ± 1.07	0.65 ± 1.07[a]	0.74 ± 1.04[b]	0.99 ± 0.93[b]	<0.001
TG (mmol/l)	1.48 (1.01-2.38)	1.38 (0.95-2.23)	1.52 (0.98-2.42)	1.47 (1.02-2.40)	1.58 (1.04-2.66)	0.427
TC (mmol/l)	5.01 ± 1.37	5.08 ± 1.57	5.14 ± 1.32	4.86 ± 1.26[a]	4.98 ± 1.29	0.023
LDL-c (mmol/l)	3.21 ± 0.95	3.25 ± 1.02	3.33 ± 0.97	3.13 ± 0.95	3.15 ± 0.82	0.013
CR (μmol/l)	68.0 (55.0-83.0)	65.0 (54.0-84.0)	68.0 (55.0-85.0)	70.0 (59.0-85.0)	68.0 (55.0-80.0)	0.708
UA (μmol/l)	359.26 ± 114.06	354.04 ± 115.65	356.31 ± 110.54	364.38 ± 121.77	362.31 ± 107.95	0.548
UACR (mg/mmol)	1.5 (0.7-5.3)	1.6 (0.8-5.0)	1.4 (0.7-6.3)	1.4 (0.7-4.3)	1.5 (0.8-6.5)	0.264
eGFR (ml/min/1.73m^2)	92.28 ± 25.73	91.88 ± 26.61	91.60 ± 25.86	90.06 ± 26.36	95.58 ± 23.78[a]	0.022
Diabetic kidney disease (n, %)[c]	336 (21.8)	76 (19.7)	77 (20.0)	81 (21.0)	102 (26.4)[a]	0.028

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; RAS, renin-angiotensin system; PWV, pulse wave velocity; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; TG, triglyceride; TC, total cholesterol; LDL-c, low-density lipoprotein cholesterol; CR, creatinine; UA, uric acid; UACR, urinary albumin to creatinine ratio; eGFR, estimated glomerular filtration rate.

[a]*P < 0.05 compared with Quartile 1 of urinary sodium.*

[b]*P < 0.01 compared with Quartile 1 of urinary sodium.*

[c]*Adjusted for age and gender.*

TABLE 3 | Stepwise regression analysis with UACR level and DKD.

Variables	UACR			DKD		
	Regression coefficient β	Standard error	*P*-value	Regression coefficient β	Standard error	*P*-value
Age (years)	-0.013	0.004	0.024	–	–	–
Gender	-0.440	0.091	<0.001	–	–	–
Smoking	–	–	–	–	–	–
Alcohol use	–	–	–	–	–	–
BMI (kg/m^2)	-0.019	0.012	0.098	–	–	–
SBP (mmHg)	0.023	0.004	<0.001	–	–	–
DBP (mmHg)	-0.009	0.005	0.043	–	–	–
PWV (cm/s)	0.001	0.0002	<0.001	0.001	0.0002	<0.001
HbA1c (%)	0.110	0.016	<0.001	0.086	0.029	0.015
HOMA-IR	–	–	–	–	–	–
TC (mmol/l)	0.125	0.029	<0.001	–	–	–
LDL-c (mmol/l)	–	–	–	–	–	–
CR (μmol/l)	1.032	0.093	<0.001	0.979	0.145	<0.001
UA (μmol/l)	0.001	0.0004	<0.001	0.001	0.0006	0.027
Urinary sodium (g/d)	0.114	0.040	0.006	0.139	0.069	0.042

UACR, urinary albumin to creatinine ratio; DKD, diabetic kidney disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PWV, pulse wave velocity; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; LDL-c, low-density lipoprotein cholesterol; CR, creatinine; UA, uric acid.

The forward stepwise regression analysis was used to obtain the determinants of UACR and DKD.

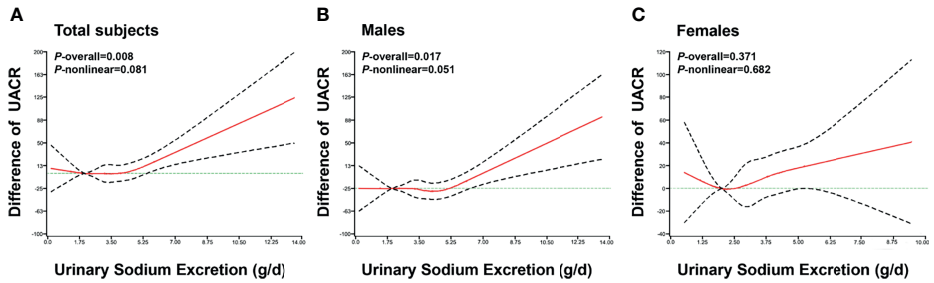

FIGURE 1 | The association of urinary sodium excretion with UACR level. The restricted cubic spline (RCS) regression was used to analyze the relationships of urinary sodium excretion (g/d) with urinary albumin to creatinine ratio (UACR) after adjusting for age, gender, BMI, smoking, alcohol consumption, DBP, RAS blocking agents, diuretics, hyperlipidemia, statin, HbA1c and antidiabetic drugs in total subjects **(A)**, males **(B)**, females **(C)**. Urinary sodium excretion was coded using an RCS function with five knots located at the 5th, 25th, 50th, 75th, 95th percentiles of the distribution of urinary sodium excretion. Y-axis represents the difference in UACR between individuals with any value of urinary sodium excretion with individuals with 2g/d of urinary sodium excretion. X-axis represents the continuous change of urinary sodium excretion. Black dashed lines are 95 percent confidence intervals.

in highest quartile of urinary sodium excretion were 1.56 times more likely to have DKD than those in the lowest quartile [OR (95% CI); 1.56 (1.07-2.27); $P = 0.020$]; urinary sodium excretion was significantly associated with the increased risks of DKD ($P < 0.05$). However, the relationship between urinary sodium excretion and the risks of DKD showed no significance, after further adjustment for HOMA-IR and PWV ($P > 0.05$). In the subgroup analyses of the associations between the risk of DKD and urinary sodium excretion levels according to the following variables: age (< 60 years/≥ 60 years), gender (male/female), hypertension (yes/no), BMI (< 24 kg/m$^{2/}$≥ 24 kg/m^2), duration of diabetes (≤ 5 years/> 5 years), HbA1c (< 9%/≥ 9%), SGLT_2i (yes/no), Diuretics(yes/no), the result indicated that the relationships of sodium excretion levels and the risk of DKD had no interaction between different subgroups (*P*-interaction > 0.05) (**Supplementary Figure 1**).

DISCUSSION

In the present study, we provided the evidence regarding the relationship between sodium intake and the presence of DKD in patients with T2DM. Our data showed that high urinary sodium excretion level was significantly associated with UACR level and increased risk of DKD, and exhibited a J-shaped relationship with UACR level. Of note, the relationship between urinary sodium excretion and risks of DKD became insignificant after further adjustment for HOMA-IR and PWV. These findings indicated that dietary sodium was associated with high risks of DKD among patients with T2DM, dependent of vascular sclerosis and insulin resistance.

Our data showed that patients with DKD had higher levels of urinary sodium excretion level than those without DKD. Prior studies showed that a survey in Japanese illustrated a reverse J-shaped relationship between daily salt intake and albuminuria in patients with T2DM (11). In contrast, Horikawa et al. reported no significant difference between sodium intake and the risk of albuminuria in patients with T2DM (12). A small cross-sectional study reported that a high-sodium diet is an independent influencing factor of microalbuminuria and renal dysfunction in 71 patients with T2DM (21). Of note, those studies used dietary recall to calculate sodium intake or estimated from a spot urine sample, which may cause a recall bias, and did not provide conclusive evidence regarding the relationship between sodium intake and the presence of DKD among the patients with T2DM. On the basis of a relative larger sample size, our study estimated sodium intake by urinary sodium excretion, and indicated that high urinary sodium was significantly associated with the risks of UACR level as well as the presence of DKD in patients with T2DM independent of several traditional risk factors. It has been proposed that high sodium intake can increase concentrations of extracellular sodium, and induce myocardial and renal fibrosis (22). Our findings suggest that monitoring sodium intake might be useful for prevention and treatment of DKD in patients with T2DM.

Our data indicated that urinary sodium level was independently correlated with UACR level as well as metabolic risk factors, such as BMI, DBP, SBP, HOMA-IR, and PWV. It has been well documented that several metabolic risk factors, including BMI and blood pressure, play a role in the development of DKD (23–25). Epidemiological study indicated that urinary sodium is linked with incidence of DKD through BMI and blood pressure (26, 27). Vedovato et al. reported that a positive correlation between higher sodium intake and albuminuria in obese adults (28). Overweight and obesity were associated with salt sensitivity and even increase glomerular filtration rate (29). Furthermore, Cardoso and colleague reported that elevated blood pressure was the main predictor of development or progression of DKD in patients with T2DM (30). Our study showed that high urinary sodium excretion was associated with the risk of DKD in patients, even adjusting for BP and BMI.

Interestingly, the relationships between urinary sodium excretion and the risks of DKD and albuminuria were no significant after further adjusting for HOMA-IR and PWV in the present study. These findings indicated that the impact of

TABLE 4 | Odds ratios (ORs) of DKD and albuminuria according to urinary sodium excretion levels.

Variables	DKD			Albuminuria		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Crude model 1						
urinary sodium (g/d)	1.09	0.96-1.23	0.189	1.19	1.02-1.41	0.033
urinary sodium (g/d)						
(Quartile 2 *vs*. Quartile 1)	0.84	0.58-1.22	0.362	0.96	0.57-1.61	0.871
(Quartile 3 *vs*. Quartile 1)	0.96	0.67-1.38	0.833	0.87	0.51-1.48	0.595
(Quartile 4 *vs*. Quartile 1)	1.29	0.91-1.82	0.154	1.17	0.71-1.92	0.550
Crude model 2						
urinary sodium (g/d)	1.12	0.99-1.27	0.083	1.23	1.04-1.46	0.019
urinary sodium (g/d)						
(Quartile 2 *vs*. Quartile 1)	1.02	0.70-1.49	0.905	1.23	0.70-2.17	0.467
(Quartile 3 *vs*. Quartile 1)	1.05	0.72-1.52	0.805	1.07	0.60-1.92	0.809
(Quartile 4 *vs*. Quartile 1)	1.44	1.00-2.06	0.049	1.53	0.88-2.65	0.129
Model 1						
urinary sodium (g/d)	1.13	1.00-1.28	0.045	1.23	1.04-1.45	0.014
urinary sodium (g/d)						
(Quartile 2 *vs*. Quartile 1)	0.98	0.69-1.40	0.906	1.09	0.65-1.81	0.747
(Quartile 3 *vs*. Quartile 1)	1.01	0.71-1.45	0.944	0.93	0.55-1.57	0.781
(Quartile 4 *vs*. Quartile 1)	1.42	1.01-2.01	0.046	1.37	0.83-2.26	0.219
Model 2						
urinary sodium (g/d)	1.19	1.04-1.35	0.009	1.24	1.05-1.47	0.014
urinary sodium (g/d)						
(Quartile 2 *vs*. Quartile 1)	1.04	0.72-1.51	0.839	1.10	0.65-1.86	0.724
(Quartile 3 *vs*. Quartile 1)	1.03	0.71-1.50	0.872	0.96	0.56-1.64	0.878
(Quartile 4 *vs*. Quartile 1)	1.62	1.13-2.33	0.009	1.37	0.82-2.31	0.234
Model 3						
urinary sodium (g/d)	1.17	1.03-1.34	0.016	1.22	1.03-1.46	0.024
urinary sodium (g/d)						
(Quartile 2 *vs*. Quartile 1)	0.99	0.68-1.45	0.976	1.03	0.61-1.76	0.903
(Quartile 3 *vs*. Quartile 1)	1.02	0.69-1.49	0.926	0.93	0.54-1.61	0.800
(Quartile 4 *vs*. Quartile 1)	1.56	1.07-2.27	0.020	1.28	0.75-2.17	0.373
Model 4						
urinary sodium (g/d)	1.13	0.99-1.30	0.076	1.19	0.99-1.43	0.068
urinary sodium (g/d)						
(Quartile 2 *vs*. Quartile 1)	0.98	0.66-1.45	0.914	1.11	0.62-2.00	0.733
(Quartile 3 *vs*. Quartile 1)	0.98	0.66-1.47	0.930	0.98	0.54-1.80	0.959
(Quartile 4 *vs*. Quartile 1)	1.45	0.98-2.15	0.062	1.29	0.72-2.33	0.395
Model 5						
urinary sodium (g/d)	1.10	0.95-1.27	0.208	1.17	0.97-1.42	0.111
urinary sodium (g/d)						
(Quartile 2 *vs*. Quartile 1)	0.80	0.53-1.22	0.309	0.96	0.52-1.78	0.900
(Quartile 3 *vs*. Quartile 1)	0.93	0.61-1.39	0.710	0.97	0.52-1.80	0.922
(Quartile 4 *vs*. Quartile 1)	1.31	0.87-1.95	0.194	1.51	0.63-2.11	0.649

DKD, diabetic kidney disease; BMI, body mass index; DBP, diastolic blood pressure; RAS, renin-angiotensin system; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; PWV, pulse wave velocity.

Crude model 1: adjusted for HOMA-IR.

Crude model 2: adjusted for PWV.

Model 1: adjusted for age, gender, BMI, smoking and alcohol consumption.

Model 2: adjusted for model 1+ DBP, RAS blocking agents, diuretics, hyperlipidemia and statin.

Model 3: adjusted for model 2+ HbA1c and antidiabetic medication.

Model 4: adjusted for model 3+ PWV.

Model 5: adjusted for model 4+ HOMA-IR.

urinary sodium excretion on DKD may be mediated by other mechanisms, such as vascular sclerosis or insulin resistance. Observational study in Japanese found that individuals with increased PWV was associated with an increased incidence of albuminuria and reduced renal function (31). The proposed mechanisms between arterial stiffness and albuminuria may be involved that increased pulsatile stress from the stiffening of large arteries elevated intrarenal pulse pressure, and lead to microvascular damage and renal insufficiency (32). In addition, insulin resistance is closely correlated with endothelial dysfunction, mild inflammation and oxidative stress, which is involved in the development of arteriosclerosis and DKD (33). Thus, our finding suggests that high urinary sodium excretion is linked with the risks of DKD through increased arteriosclerosis and insulin resistance.

Additionally, our data indicated that 2 g/d of sodium excretion were associated with lower UACR levels, which is consistent with the recommendation by WHO (20). Recently,

ADA also recommended a reduction to <2 g/d sodium (5 g/d salt) in patients with diabetes (34). However, guidelines from UK and USA recommended sodium intake reduction that is based on the relationship between high sodium intake and the risks of hypertension and CVD (35, 36). Previous study reported that lower 24-h urinary sodium excretion (<150 mmol Na/day) was inversely associated with increased all-cause mortality (37), which may be higher than the recommended intake (5 g/d salt). Thomas et al. showed that the lowest sodium excretion was associated with the highest cumulative incidence of ESRD in the subgroup of 424 patients with macroalbuminuria (38). Additionally, this study also showed that both high and low sodium intake were associated with adverse mortality outcomes. Our study illustrated that there was a J-shaped relationship between sodium intake and UACR level in patients with T2DM rather than monotonic linear relations. This result also suggested that both higher and lower sodium intake were associated with increased urinary albumin excretion and might cause damage to renal function, underscoring the importance of moderate restriction of sodium intake for reducing the risk of DKD.

This study has the following limitations. First, the study was a cross-sectional design. Causality between urinary sodium excretion and the presence of DKD cannot be determined. It is necessary to determine the association of sodium intake and renal outcomes with long term follow-up period in the prospective cohort study. Second, we used morning fasting urine samples instead of 24 h urine samples for estimating sodium excretion in outpatients.

CONCLUSION

In conclusion, our data demonstrated that high urinary sodium excretion was associated with high risk of DKD among patients with T2DM, dependent of vascular sclerosis and insulin resistance. Our findings suggest that moderate restriction of sodium intake might be benefit for reducing the risk of DKD. Further study needs to determine the association of sodium intake and the presence of DKD in the prospective studies.

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 study protocol was approved by the Institutional Review Board of Nanfang Hospital of Southern Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YH, HZ, and YX contributed to conception and design of the study. HZ and YX supervised the study. DG, PZ, DL and LY organized the database. YH, JL and Jy L performed the statistical analysis. YH wrote the first draft of the manuscript. YH, WL and JL wrote sections of the manuscript. 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.2021.772073/full#supplementary-material

Supplementary Figure 1 | Odds ratios of DKD in different subgroups. Forest plot of odds ratios of DKD according to urinary sodium excretion levels in different subgroups. Patients were categorized according to the following variables: age (<60 years/≥60 years), gender (male/female), hypertension (yes/no), BMI (< 24 kg/m^2/≥ 24 kg/m^2), duration of diabetes (≤ 5 years/> 5 years), HbA1c (< 9%/≥ 9%), SGLT_2i(yes/no), Diuretics(yes/no).

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Bayesian Age-Period-Cohort Prediction of Mortality of Type 2 Diabetic Kidney Disease in China: A Modeling Study

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Background: The burden of type 2 diabetic kidney disease (DKD) continues to rise in China. We analyzed time trends in DKD mortality and associations with age, period, and birth cohort from 1990 to 2019, made projections up to 2030, and examined the drivers of deaths from DKD.

Methods and Findings: The number of DKD deaths in China from 1990 to 2019 was obtained from the GBD 2019. We used age-period-cohort modeling to estimate age, period, and cohort effects in DKD mortality between 1990 and 2019. We calculated net drift (overall annual percentage change), local drift (annual percentage change in each age group), longitudinal age curves (expected longitudinal age-specific rates), period, and cohort relative risks. We used Bayesian age-period-cohort analysis with integrated nested Laplace approximations to project future age-specific DKD death cases from 2020 to 2030. We used a validated decomposition algorithm to attribute changes in DKD deaths to population growth, population aging, and epidemiologic changes from 1990 to 2030. From 1990 to 2019, the age-standardized mortality rate of DKD in China was relatively stable, but the absolute number of DKD deaths showed a noticeable increasing trend. The overall annual percentage change (net drift) was -0.75% (95% confidence interval, CI: -0.93 to -0.57) for males and -1.90% (95% CI, -2.19 to -1.62) for females. The age-specific annual percentage changes (local drifts) were below zero in all age groups from 1990 to 2019 except for males aged above 65 to 69 years, and for females aged above 70 to 74 years. The risk of DKD deaths increased exponentially with age for both sexes after controlling for period deviations. The Bayesian age-period-cohort analysis projects that there would be 88,803 deaths from DKD in 2030, increased by 224.2% from 1990. Despite a decrease in age-specific DKD death rates, the reduction would be entirely offset by population aging.

Conclusions: Although China has made progress in reducing DKD deaths, demographic changes have entirely offset the progress. The burden of DKD deaths is likely to continue

increasing. Our findings suggest that large-scale screening is imperative for DKD control and prevention, particularly for high-risk groups.

Keywords: type 2 diabetic kidney disease, mortality, age-period-cohort modeling, projection, demographic change

INTRODUCTION

Type 2 diabetic kidney disease (DKD) is a common microvascular complication of type 2 diabetes mellitus (T2DM), occurs in approximately 20%-30% of diabetic patients, and is one of the leading causes of end-stage renal disease (ESRD) (1–3). DKD manifests as albuminuria, impaired glomerular filtration rate (GFR), or both, and even mild albuminuria and reduced GFR are associated with a significantly increased risk of cardiovascular disease and death (4, 5). In addition, patients with DKD-ESRD have a high mortality rate than non-DKD ESRD patients (6). Epidemiological studies have suggested that DKD has become the leading cause of chronic kidney disease (CKD) in the pre-dialysis CKD population in China, surpassing glomerulonephritis, and therefore will become the leading cause accounting for dialysis in the near future (7). The prevalence of DKD increases in direct proportion to the prevalence of T2DM (8); thus, the burden of DKD in China is likely to continue to increase as the prevalence of T2DM has risen sharply (7).

Although there has been an increasing trend in DKD burden in China across time (7, 9–11), the approaches used in previous studies fail to differentiate the relative contribution of period and cohort effects to overall time trends, which hinders us from evaluating the success of earlier policy interventions. We aimed to address this knowledge gap by evaluating how age, calendar period, and birth cohort are associated with increased mortality from DKD in China using an age-period-cohort analysis. Age effects are the changes related to the biological and social processes of aging specific to an individual. Period effects are caused by external factors that affect all age groups within a given calendar time. Cohort effects result from the unique experience or exposure of a group of subjects (the cohort) at different times (12, 13).

Estimation of future DKD mortality trends is vital for DKD control planning. We used Bayesian age-period-cohort analysis to predict future DKD deaths, which has been extensively used to predict the future burden of many diseases (14, 15). To analyze the drivers of DKD deaths, we used a validated decomposition algorithm (16) to attribute changes in the number of DKD deaths to population growth, population aging, and epidemiological changes in DKD. The findings of this study will improve our understanding of the time trends of DKD burden in China and identify potential drivers for the changes in DKD deaths, which may help guide public health policy, resource allocation, and the design of screening programs.

METHODS

Study Data

We obtained China DKD mortality data from the Global Burden of Disease (GBD) Study 2019, which is a multinational collaborative study that estimates disease burden in 204 countries and territories worldwide (17, 18). The methods used in GBD 2019 have been reported in detail elsewhere (17–19). In brief, GBD 2019 used vital registration and verbal autopsy data to model mortality due to chronic kidney disease (CKD) (17). The Bayesian geospatial regression model was used to increase the comparability of mortality data sources that used location-specific covariates to create smoothed time trends. Data from the ESRD registry were used to estimate five causes of CKD: type 1 diabetes, type 2 diabetes, glomerulonephritis, hypertension, and a residual category of other and unspecified causes. The DKD data analyzed in this paper refer to the data on CKD due to type 2 diabetes. An epidemiologic state-transition disease modeling tool was used to produce consistent estimates by location, year, age, and sex. These adjusted proportions were applied to the parent CKD regression model to obtain type-specific estimates of CKD. As the data were publicly available and data were aggregated and de-identifiable, institutional review board approval and informed consent were not needed.

Statistical Analysis

We used the age-period-cohort framework to estimate the following parameters: (1) net drift, representing the overall log-linear trend by period and birth cohort, indicating the overall annual percentage change of the expected age-adjusted rate; (2) local drifts, representing the log-linear trends for each age group by period and birth cohort, indicating the annual percentage change of the expected age-specific rate over time; (3) longitudinal age curve, showing the expected age-specific rates adjusted for period effects in reference cohort; (4) period (or cohort) rate ratios (RR), representing the ratio of age-specific rates in each period (or cohort) relative to the reference one.

For age-period-cohort analyses, we arranged the DKD mortality and population data into consecutive 5-year periods from 1990 to 2019, and successive 5-year age intervals from 15-19 years to 95 plus. The birth cohort was defined using the difference between the medium value of the age interval and the period interval. We obtained the estimated parameters by the age-period-cohort Web Tool provided by the National Cancer Institute (20). For relative rate measurements, the reference period interval was from 2000 to 2004, and the reference birth cohort interval was from 1945 to 1949. We used the Wald chi-square test to test the significance of the estimable parameters and functions. All statistical tests were two-sided.

We used the Bayesian age-period-cohort analysis with integrated nested Laplace approximations to project the future age-specific number of death cases from DKD from 2020 to 2030 (21), which shows better coverage and precision than other prediction methods (22). Based on the assumption that the effects of age, period and cohort adjacent in time are similar,

the Bayesian inference in age-period-cohort model applies the second-order random walk for smoothing priors of age, period, and cohort effects and to project posterior mortality rates. The integrated nested Laplace approximations are used with this Bayesian age-period-cohort model to approximate the marginal posterior distributions avoiding any mixing and convergence issues introduced by Markov chain Monte Carlo sampling techniques traditionally used in the Bayesian approach. We conducted the Bayesian age-period-cohort analysis using R-package BAPC (version 0.0.34). We provided additional details in **Text S1**. The population predictions for China were taken from the 2019 revision of the United Nations (UN) World Population Prospects and were used to estimate China's population in 2020 and beyond (23).

To analyze the drivers of the changes in the number of DKD deaths from 1990 to 2030, we used a newly developed decomposition method to attribute changes in the total number of DKD deaths to population growth, population aging, and age-specific changes in DKD mortality between 1990 and each subsequent year from 1991 to 2030 (16, 24). Briefly, this decomposition method has considered the 2-way and 3-way interactions of the three components and is robust to the choice of the decomposition order of the three factors, and the selection of the reference year compared to previous decomposition methods (25, 26). Details about the decomposition method were described elsewhere (16, 24) and in the **Text S2**. This method has been used to quantify the impact of population aging on mortality for 195 countries or territories and 169 causes of deaths (24), and to quantify the demographic and epidemiologic drivers for the impacts of air pollution and high sodium intake (27–29). We calculated the absolute and relative contributions of the three drivers to the change in the number of DKD deaths. The absolute contribution was the number of attributed DKD deaths, while the relative contribution was estimated as the number of attributed DKD deaths divided by the total DKD deaths in 1990×100%. A positive contribution indicates an increase in total DKD deaths, while a negative contribution indicates a decrease in total DKD deaths. The age-specific changes in DKD deaths refer to epidemiologic changes, which include all differences in mortality that cannot be explained by population growth and population aging (30), such as new treatments or medications for DKD. The net changes in these three components are equal to the difference in the total number of observed deaths. We performed statistical analyses with R software (Version 3.6.3, R core team).

RESULTS

Trends in DKD Mortality

In 2019, there were 63,354 (95% UI: 49,787 to 77,280) DKD deaths in China, and the age-standardized mortality rate of DKD was 3.6 (95% UI: 2.8 to 4.3) per 100,000. Between 1990 and 2019, the total number of DKD deaths increased dramatically from 13,269 (95% UI: 9,998 to 16,930) in 1990 to 32,296 (95% UI: 24,275 to 41,393) in 2019 for males and from 14,144 (95% UI: 10,909 to 17,470) in 1990 to 31,058 (95% UI: 23,720 to 38,907) in 2019 for females (**Figure 1A**). On the contrary, the age-standardized mortality rate of DKD was relatively stable for both sexes (**Figure 1B**).

Age-Period-Cohort Analysis

Net drift represents the overall annual percentage change across the study period (**Figure 2**). We found marked sex differences in net drift with -0.75% (95% confidence interval, CI: -0.93% to -0.57%) for males and -1.90% (95% CI: -2.19% to -1.62%) for females, reflecting less improvement in reduction of DKD mortality for males than for females from 1990 to 2019. Local drift reflects additional age-specific variations in DKD mortality trends (**Figure 2**). Values lie predominantly below 0 for both sexes for most age groups, indicating improvements in reducing DKD mortality. The exceptions were males aged above 65 to 69 and females aged above 70 to 74, indicating increased mortality from DKD.

For both sexes, in the same birth cohort, the risk of death from DKD showed an accelerated increase with age. We performed a curve estimation for the longitudinal age curves and found that both sexes showed an exponential distribution (**Figure 3**). The relationship between age and mortality rate can be expressed as mortality rate=$0.023\times e^{0.097\times age}$ for males (R-squared=0.997) and mortality rate=$0.056\times e^{0.081\times age}$ for females (R-squared=0.997), where age is the median age of each age interval. These indicated that the mortality risk of DKD was 128-fold higher for males and 57-fold higher for females aged 75 to 79 years compared to the corresponding males and females aged 20 to 24, respectively.

The period (cohort) relative risks are the ratio of age-specific rates in each period (cohort) relative to the reference period (cohort). We found decreased period relative risks for both sexes, with a more quickly decreasing trend for females than for males during the whole study period after adjusting for age and birth cohort (**Figure 4**). Cohort relative risks were also found in similar patterns for both sexes, starting to decline after 1935 for females and after 1945 for males and then declining more rapidly for females (**Figure 5**). In addition, using the specific results of Wald tests, we found cohort and period effects for both sexes, and the net drifts and local drifts were all statistically significant ($p<0.05$) (**Table S1**).

DKD Mortality Projection

We next conducted a Bayesian age-period-cohort analysis to project future mortality trends for DKD in China. Our results showed that the total number of deaths from DKD in China would continue to increase, with 88,803 deaths from DKD in China by 2030 (**Table S2**). However, there were significant differences in the distribution of DKD deaths across age groups, with more occurring in the older age groups and a continued increase in the older age groups (above 60 years), but a decreasing trend in the younger age groups (under 50 years).

Decomposition Analysis

Finally, we conducted a demographic decomposition analysis to identify DKD mortality drivers from 1990 to 2030. Our results showed that demographic factors drove the increasing trend in

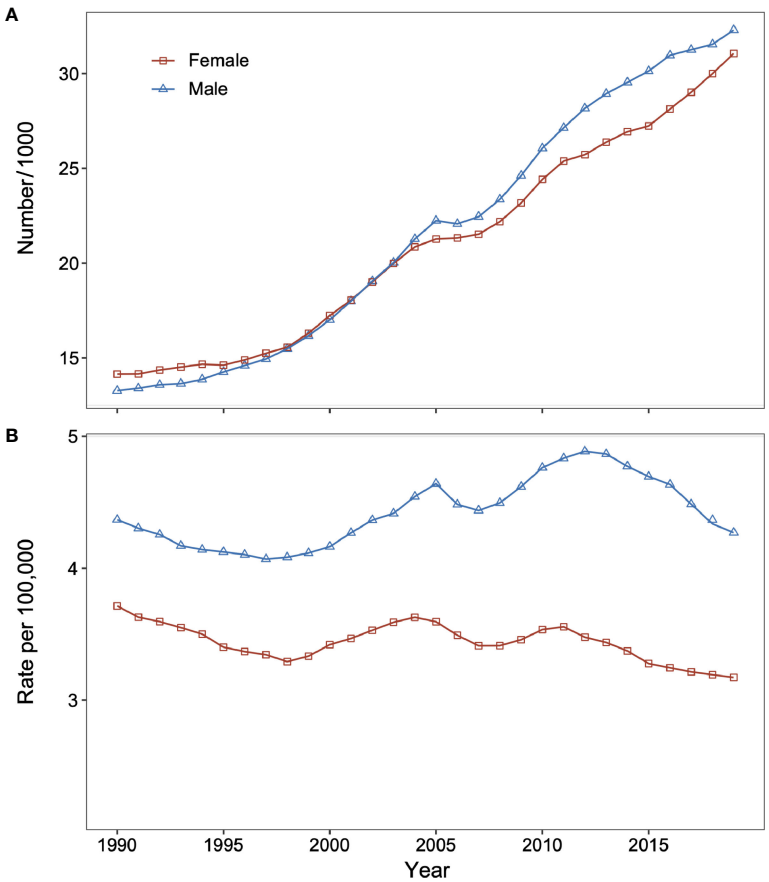

FIGURE 1 | Changes in type 2 diabetic kidney disease (DKD) mortality and number of deaths for males and females in China from 1990 to 2019. **(A)** Number of DKD deaths for males and females. **(B)** Age-standardized death rate of DKD for males and females. DKD, type 2 diabetic kidney disease.

the number of DKD deaths in China, with population aging playing a dominant role, especially after 2010 (**Table 1**, **Figure 6**).

There were 35,942 additional DKD deaths in China in 2019 from 1990, an increase of 131.2%. The increase was driven by changes in the number of DKD deaths due to population aging (90.2% increase from 1990) and population growth (53.5% increase from 1990). Our projection suggests that the increasing trend in the number of DKD deaths will continue. By 2030, China will have 224.2% more DKD deaths than in 1990, with a contribution of 173.9% increase in deaths due to population aging and a 74.9% increase due to population growth, despite a 24.7% decrease in age-specific death rates (**Table 1**, **Figure 6**).

DISCUSSION

In this study, we estimated age, period, and cohort effects in DKD mortality between 1990 and 2019 using age-period-cohort analyses, predicted DKD deaths from 2020 to 2030 using Bayesian age-period-cohort analysis with integrated nested Laplace approximations and decomposed the main drivers of the changes in DKD deaths from 1990 to 2030. We found a decreasing trend in both cohort and period effects for DKD deaths in China, suggesting the success of earlier policies in reducing DKD deaths. We estimated that by 2030, DKD deaths would increase dramatically by 224.2% from 1990, driven primarily by population aging, which completely offset the reduction in DKD deaths due to epidemiological changes.

Although previous studies have shown an association between DKD mortality and age (1, 8), we quantitatively demonstrated an exponential increase in DKD mortality with age after adjusting for period and cohort effects. This age effect may be partly due to the more unsatisfactory treatment outcome and prognosis of DKD with increasing age. At the same time, the higher all-cause mortality may explain the higher DKD morality for males than for females (31).

Improvements in medical conditions are the main reason for the monotonic decline between the period and DKD mortality. Fast urbanization and advances in primary health care in China over the last three decades have promoted the availability, accessibility, and affordability of health care services, especially as the Chinese government has continued to improve the health care system in recent years, resulting in more than 99.9% of the poor population participating in basic health insurance, these

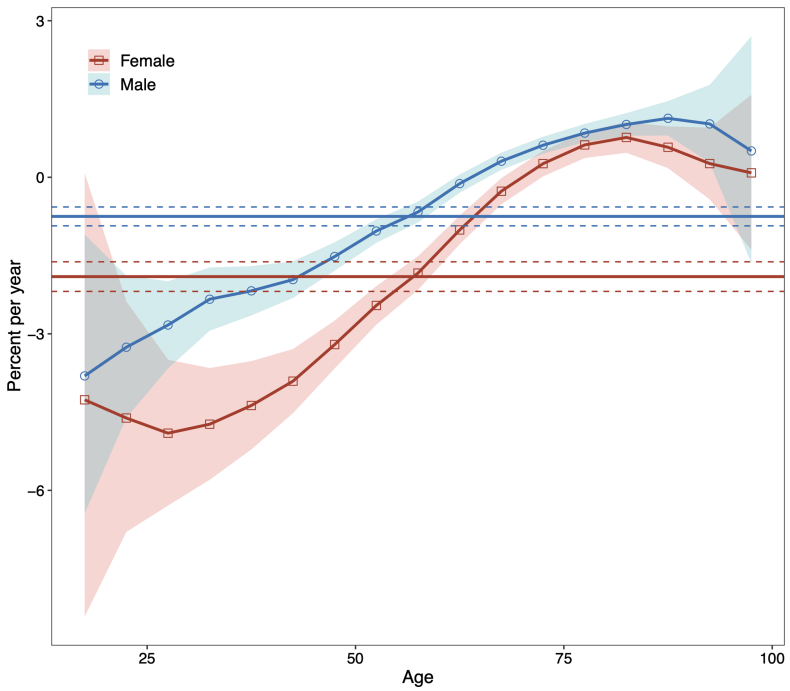

FIGURE 2 | Local drifts with net drift values for males and females for type 2 diabetic kidney disease (DKD) mortality in China from 1990 to 2019. The horizontal solid lines are the net drifts, and the dashed lines showed their 95% confidence intervals. The solid line of the curve are the local drifts and the shaded area indicate their 95% confidence intervals. DKD, type 2 diabetic kidney disease.

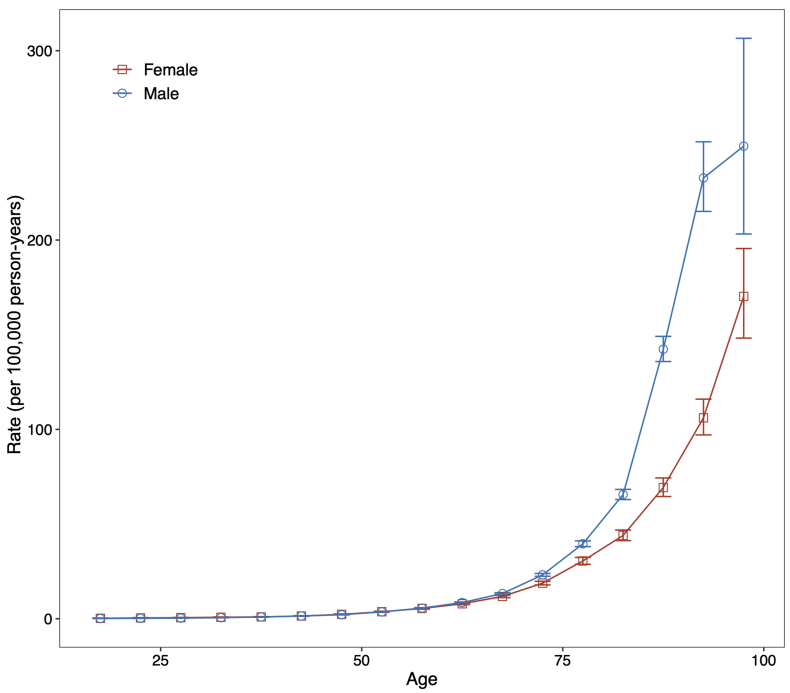

FIGURE 3 | Fitted longitudinal age curves of type 2 diabetic kidney disease (DKD) mortality (per 100,000 person-years) and the corresponding 95% confidence interval for males and females. DKD, type 2 diabetic kidney disease.

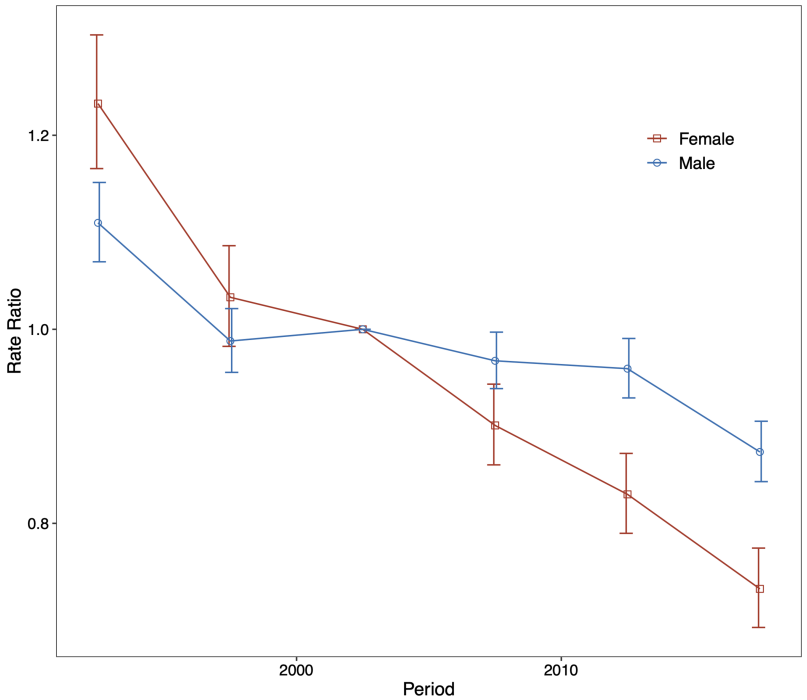

FIGURE 4 | Relative risk of each period compared with the reference period (2000–2004) adjusted for age and nonlinear cohort effects and the corresponding 95% confidence interval for males and females.

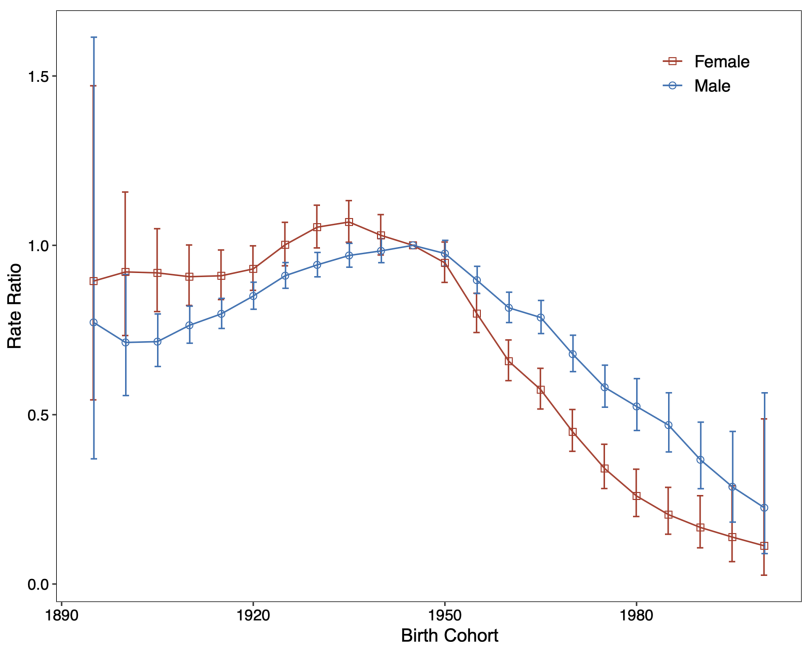

FIGURE 5 | Relative risk of each cohort compared with the reference cohort (cohort 1945–1949) adjusted for age and nonlinear period effects and the corresponding 95% confidence interval.

TABLE 1 | Contribution of changes in population aging, population growth, and age-specific death rate of type 2 diabetic kidney disease (DKD) to the net change of DKD deaths in China from 1991 to 2030, using 1990 as the reference year.

Year	Due to population aging, n (%)	Due to population growth, n (%)	Due to age-specific death rate, n (%)	Net change n (%)
1991	526 (1.9)	386 (1.4)	-748 (-2.7)	164 (0.6)
1992	1030 (3.8)	842 (3.1)	-1291 (-4.7)	581 (2.1)
1993	1493 (5.4)	1314 (4.8)	-1987 (-7.3)	820 (3.0)
1994	1913 (7.0)	1745 (6.4)	-2390 (-8.7)	1267 (4.6)
1995	2237 (8.2)	2102 (7.7)	-2818 (-10.3)	1521 (5.6)
1996	2889 (10.5)	2646 (9.7)	-3480 (-12.7)	2055 (7.5)
1997	3483 (12.7)	3040 (11.1)	-3792 (-13.8)	2732 (10.0)
1998	4026 (14.7)	3387 (12.4)	-3696 (-13.5)	3716 (13.6)
1999	4462 (16.3)	3827 (14.0)	-3240 (-11.8)	5049 (18.4)
2000	4827 (17.6)	4430 (16.2)	-2428 (-8.9)	6829 (24.9)
2001	5519 (20.1)	5008 (18.3)	-1785 (-6.5)	8741 (31.9)
2002	6049 (22.1)	5749 (21.0)	-1183 (-4.3)	10615 (38.8)
2003	6536 (23.9)	6596 (24.1)	-534 (-1.9)	12598 (46.0)
2004	7037 (25.7)	7425 (27.1)	244 (0.9)	14707 (53.7)
2005	7521 (27.5)	8083 (29.5)	467 (1.7)	16071 (58.7)
2006	8325 (30.4)	8563 (31.3)	-863 (-3.2)	16025 (58.5)
2007	9197 (33.6)	9017 (32.9)	-1575 (-5.7)	16639 (60.7)
2008	10175 (37.1)	9502 (34.7)	-1441 (-5.3)	18236 (66.6)
2009	11150 (40.7)	10033 (36.6)	-768 (-2.8)	20416 (74.5)
2010	12178 (44.5)	10621 (38.8)	260 (1.0)	23059 (84.2)
2011	13608 (49.7)	11148 (40.7)	319 (1.2)	25075 (91.5)
2012	14914 (54.4)	11612 (42.4)	-21 (-0.1)	26505 (96.8)
2013	16162 (59.0)	12077 (44.1)	-292 (-1.1)	27947 (102.0)
2014	17329 (63.3)	12498 (45.6)	-686 (-2.5)	29140 (106.4)
2015	18367 (67.0)	12862 (47.0)	-1219 (-4.5)	30010 (109.6)
2016	20027 (73.1)	13338 (48.7)	-1685 (-6.2)	31680 (115.6)
2017	21560 (78.7)	13749 (50.2)	-2485 (-9.1)	32825 (119.8)
2018	23112 (84.4)	14173 (51.7)	-3138 (-11.5)	34147 (124.7)
2019	24701 (90.2)	14644 (53.5)	-3403 (-12.4)	35942 (131.2)
2020	26392 (96.3)	15121 (55.2)	-3553 (-13.0)	37960 (138.6)
2021	28563 (104.3)	15684 (57.3)	-3802 (-13.9)	40446 (147.6)
2022	30661 (111.9)	16230 (59.2)	-4079 (-14.9)	42812 (156.3)
2023	32655 (119.2)	16755 (61.2)	-4376 (-16.0)	45034 (164.4)
2024	34514 (126.0)	17254 (63.0)	-4702 (-17.2)	47066 (171.8)
2025	36237 (132.3)	17725 (64.7)	-5045 (-18.4)	48917 (178.6)
2026	38754 (141.5)	18342 (67.0)	-5371 (-19.6)	51725 (188.8)
2027	41185 (150.3)	18934 (69.1)	-5729 (-20.9)	54390 (198.6)
2028	43493 (158.8)	19498 (71.2)	-6090 (-22.2)	56901 (207.7)
2029	45648 (166.6)	20030 (73.1)	-6442 (-23.5)	59236 (216.2)
2030	47650 (173.9)	20528 (74.9)	-6768 (-24.7)	61410 (224.2)

DKD, type 2 diabetic kidney disease.

initiatives have greatly improved the treatment of DKD, for instance, the affordability and accessibility of dialysis have been greatly improved (11, 32), thus significantly reducing the mortality of DKD.

The decline in the cohort effects of DKD mortality may be due to improved medical conditions, with more deaths due to DKD among those born before the 1950s and a gradual downward trend in DKD deaths in the post-1950 cohort. The lack of nutritional conditions in early life may be a risk factor for the high incidence of diabetes and kidney disease in adulthood (33). At the same time, social unrest in China before 1950 may have contributed to nutritional deficiencies in early life. In addition, better education and better awareness of diabetes in successive generations may have played a partial role (34). It is worth pointing out that although the period and cohort effects can be estimated as period relative risk and cohort relative risk, respectively, it is not appropriate to interpret them completely separately (12, 13, 35), because there is an interaction between the two.

Our study showed that the number of DKD deaths in China had increased significantly over the past three decades. In contrast, the age-standardized DKD mortality rate has fluctuated only marginally. The inconsistency reflects the vital role that demographic change plays in DKD deaths. While significant improvements in DKD diagnosis, treatment, and management techniques in recent decades, accompanied by more and better healthcare professionals, have played a key role in reducing deaths from DKD, however, these advances have been offset by changes in demographics and population size. Population aging has become the main dominant driver in the absolute number of DKD deaths in China, and this trend is set to continue as the population continues to age. In contrast, the role of population growth is relatively weak. These suggest that China needs to allocate healthcare resources to cope with

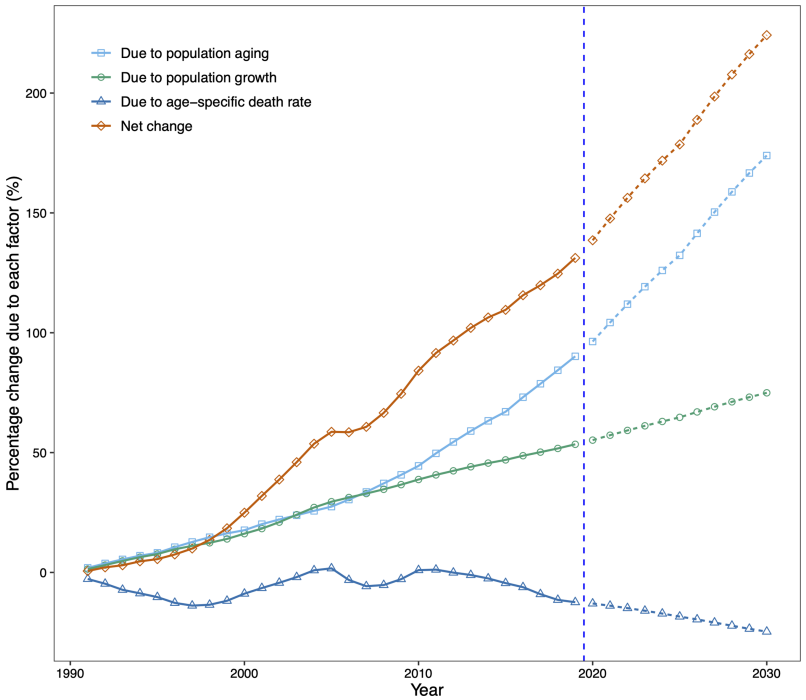

FIGURE 6 | Contribution of changes in population aging, population growth, and age-specific type 2 diabetic kidney disease (DKD) death rate to changes in number of DKD deaths from 1991 to 2030 for both sexes in China, using 1990 as the reference year. Data in the right of the blue dashed line were the decomposition based on the projected data. DKD, type 2 diabetic kidney disease.

the changes in healthcare needs brought about by an aging population.

It is noteworthy that the prevalence of risk factors for DKD in China, particularly diabetes, is not well controlled. For example, the prevalence of diabetes (a major DKD risk factor) has increased rapidly in the Chinese adult population since 1990 (36). However, we also note that effective and increasingly common measures to control blood glucose and large-scale screening for chronic kidney disease may alleviate the burden of diabetes to some extent and further alleviate the burden of DKD (7, 37).

The present study has some limitations. First, the Bayesian age-period-cohort requires fixed age and period intervals, however the age group of 95 and plus recorded in the GBD 2019 database might vary by years. We expect the varying 95 and plus age interval should not materially affect our results given the proportion of people aged over 100 years is small. Second, GBD 2019 includes limited sources from a small number of countries (8, 18), and only used data from the end-stage renal registry to model the proportion of deaths due to CKD, without considering other causes of DKD deaths such as nephrotic syndrome, so there is likely to be some uncertainty in the DKD estimates in China (18). Third, there may be some uncertainty in the UN projections of the size and distribution of China's population, which could affect the population-based analysis, such as decomposition and projection. Fourth, we contributed the increase in DKD deaths into population growth, population aging, and epidemiologic changes and did not further examine other factors that could influence DKD deaths, such as age at onset age of diabetes and DKD, diabetic duration, and blood control, due to the lack of available data in the GBD database.

CONCLUSION

The burden of DKD deaths in China is likely to continue increasing. Although China has made progress in reducing DKD deaths, demographic changes have entirely offset the progress, primarily driven by population aging. Our findings suggest the urgency of improving health systems to meet the health needs of older adults, and the importance of large-scale screening and risk factor control for DKD control and prevention.

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.

AUTHOR CONTRIBUTIONS

XW, JD, and SS designed the study, wrote, reviewed, and edited the manuscript. LL and WC reviewed and

contributed to edit the manuscript. XW, JD, and LL researched and analyzed data. JD is the guarantor of this work. 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.2021.767263/full#supplementary-material

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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 mRNA Signatures as Predictors of Renal Function Decline in Patients With Biopsy-Proven Diabetic Kidney Disease

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The clinical manifestations of diabetic kidney disease (DKD) are more heterogeneous than those previously reported, and these observations mandate the need for the recruitment of patients with biopsy-proven DKD in biomarker research. In this study, using the public gene expression omnibus (GEO) repository, we aimed to identify urinary mRNA biomarkers that can predict histological severity and disease progression in patients with DKD in whom the diagnosis and histologic grade has been confirmed by kidney biopsy. We identified 30 DKD-specific mRNA candidates based on the analysis of the GEO datasets. Among these, there were significant alterations in the urinary levels of 17 mRNAs in patients with DKD, compared with healthy controls. Four urinary mRNAs—*LYZ, C3, FKBP5*, and *G6PC*—reflected tubulointerstitial inflammation and fibrosis in kidney biopsy and could predict rapid progression to end-stage kidney disease independently of the baseline eGFR (tertile 1 *vs*. tertile 3; adjusted hazard ratio of 9.68 and 95% confidence interval of 2.85–32.87, $p < 0.001$). In conclusion, we demonstrated that urinary mRNA signatures have a potential to indicate the pathologic status and predict adverse renal outcomes in patients with DKD.

Keywords: diabetic kidney disease, mRNA, urine, biomarker, renal pathology

INTRODUCTION

Diabetic kidney disease (DKD) is the leading cause of end-stage kidney disease (ESKD) globally, including in Korea (1). The diagnosis of DKD is traditionally based on the assessment of persistent albuminuria and decline of estimated glomerular filtration rate (eGFR); renal biopsy is not routinely performed as the natural course of DKD has previously been described as predictable (2, 3). However, it is difficult to unify the clinical spectrums of DKD as a simple and predictable disease due to the complexity of its pathogenesis and its various progression patterns (4). A large epidemiological study has revealed the decreasing prevalence of albuminuria and increasing prevalence of eGFR in DKD over the last 3 decades (5). Moreover, non-diabetic renal disease (NDRD) is frequently detected among diabetic patients who have undergone renal biopsy, raising a concern that patients with

clinically diagnosed DKD may have associated NDRD (6–10). Thus, identifying patients in whom DKD diagnosis has been confirmed through kidney biopsy is an essential prerequisite for the successful discovery of relevant biomarkers. Unfortunately, this approach has rarely been used in the field of DKD research, partially justifying the reason for the validation failure of previously identified DKD biomarkers (11). Nonetheless, the incidence of biopsy-proven DKD has been increasing over the past decades (12).

The Renal Pathology Society has proposed pathologic classifications of DKD based on glomerular, tubulointerstitial, and vascular compartments (13). Several studies have consistently shown that this classification system is valuable in predicting a subsequent decline in kidney function (14–18). Nonetheless, its relevance is largely limited in clinical practice since most patients suspected to have DKD do not undergo renal biopsy. Meanwhile, non-invasive biomarkers that can reflect intrarenal pathology might be useful in predicting the renal prognosis in patients with DKD and absence of kidney biopsy. In this regard, we have previously identified that urinary CXCL16 and endostatin, indicative of the degree of tubulointerstitial fibrosis, successfully predicted poor renal outcomes in patients with biopsy-proven advanced DKD (18).

Over the past decade, omics technologies have been increasingly applied for the identification of biomarkers, including in kidney diseases (19). These web-based data platforms allow us to generate molecular profiles and assess the relevance of biological pathways, networks, potential targets, and biomarkers in diseases. In this study, through utilization of the public Gene Expression Omnibus (GEO) repository, we aimed to identify urinary mRNA biomarkers that can predict disease progression in patients with biopsy-proven DKD.

MATERIALS AND METHODS

Patient Selection and Study Design

An overview of the study design and patient recruitment strategy is illustrated in **Figure 1**. We retrospectively screened 155 patients with biopsy-proven isolated DKD without NDRD at Kyung Hee Medical Center and Kyung Hee University Hospitals at Gangdong from January 2010 to March 2020. The patients were excluded in the following circumstances: unavailability of urine sample, refusal for sample collection, or biopsy samples containing <10 glomeruli. Finally, we enrolled 83 patients with DKD whose urine samples were available. We also recruited 19 patients with combined NDRD and DKD and 32 healthy controls. Individuals fulfilling all the following criteria were included as healthy controls: 1) normal renal function (eGFR > 90 ml/min/1.73 m^2), 2) absence of proteinuria or hematuria, and 3) absence of diabetes or hypertension. Indications for renal biopsy in diabetic patients are described elsewhere (6).

The baseline characteristics and laboratory parameters of the enrolled patients were collected at the time of renal biopsy. Renal function was assessed by eGFR, calculated using the Chronic Kidney Disease Epidemiology Collaboration formula (20). Renal outcomes were defined as progression to ESKD requiring renal replacement therapy or transplantation.

Ethics Statement

This study was conducted according to The Code of Ethics of the World Medical Association (Declaration of Helsinki), and was reviewed and approved by the local ethics committee (IRB no. KHNMC2021-01-054-003). Informed consent was obtained from the study participants.

Pathologic Diagnoses of Diabetic Kidney Diseases and Non-Diabetic Renal Disease

All biopsy specimens were processed by standard methods and routinely examined by light microscopy, immunofluorescence, and electron microscopy. The diagnosis of DKD was made and categorized according to the pathologic classification of the Renal Pathology Society (13). In brief, this classification system includes five histologic parameters: glomerular classification, interstitial fibrosis and tubular atrophy (IFTA), interstitial inflammation, arterial hyalinosis, and arteriosclerosis. The diagnosis of NDRD accompanied with DKD was made when

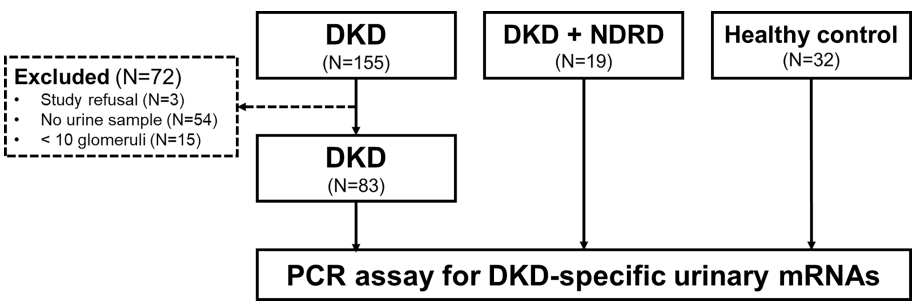

FIGURE 1 | Flowchart of participant selection. We first screened 155 patients with diabetic kidney disease (DKD) whose diagnoses were confirmed by kidney biopsy. Among these, 83 patients with availability of urine samples were enrolled in this study. We also recruited 19 patients exhibiting both DKD and non-diabetic renal disease (NDRD) and 32 healthy individuals as control groups. Urinary cell pellets from the participants were collected and analyzed for measurement of the levels of DKD-specific mRNA candidates selected based on the metanalysis of the public GEO repository. DKD, diabetic kidney disease; NDRD, non-diabetic renal disease; PCR, polymerase chain reaction.

the kidney biopsy tissue exhibited typical features of both DKD and other glomerulopathies.

Selection of Diabetic Kidney Disease-Specific mRNA Candidates

Upon searching through the GEO database using the keywords "diabetic kidney disease" and "diabetic nephropathy," we found two data sets (GSE104948 and GSE104954) with the whole gene expression profiles of both DKD patients and corresponding healthy controls. The meta-analysis of the two data sets was performed by GeneMeta R package that follows the approach of Choi et al. (21). Random effects models were used for the meta-analysis. The false discovery rates (FDRs) were obtained from 1,000 permutations, and the effective fold changes were calculated as the average fold changes of two data sets weighted by the number of samples. Those with fold change ≥2 or ≤0.5, and FDR <0.001 were selected as the mRNA candidates in each data set.

Collection of Urinary Samples and Measurements of Urinary mRNA Levels

Urine sample collection, processing, and storage was performed in an aseptic manner by an experienced technician to avoid cross-contamination. Mid-stream urine samples were collected on the day of renal biopsy or at the time of visit for medical checkup and were centrifuged at 2,000 g for 20 min at room temperature. Cell pellets were separated on clean benches, subsequently transferred into RNA (Invitrogen, Carlsbad, CA), and stored at -80°C until required. All these processes were performed immediately after urine sample collection; therefore, the urine samples were stored within 1 hour of collection. Total RNA was extracted using the PureLinkTM RNA Mini Kit (Invitrogen), according to the manufacturer's recommendations. The amount of total RNA (ug) was measured using a NanoDrop® ND-2000 UV spectrophotometer (Thermo Scientific, Waltham, MA), cDNA synthesis was performed with the total RNA using M-MLV RT enzyme (200 U/μl; Mbiotech, Inc., Seoul, Korea), and the levels of gene expressions using each target primer and SYBR Green Master Mix (Applied Biosystems, Foster city, CA) were measured on ABI StepOne real-time polymerase chain reaction system (Applied Biosystems), as previously described (22). Each mRNA level was normalized by 18S rRNA used as an endogenous control for the 2-ΔΔCt method, and then $\log_{10}$-transformed to reduce deviation.

Statistical Analyses

All statistical analyses were performed with SPSS for Windows, version 20.0 (IBM, Armonk, NY). Baseline characteristics and clinical parameters are expressed as the mean ± standard deviation or as number of patients and percentage. Analysis of variance and Bonferroni post-hoc test was used for comparisons of urinary mRNA levels among different groups. The combined scores of mRNA signatures were determined by calculating the predicted probabilities of ESKD progression for each patient using logistic regression analysis. Patients were then divided into tertiles according to their values of calculated probability. Kaplan–Meier curves were generated to illustrate the cumulative probabilities of renal outcomes, and the Cox proportional hazards model was used for the multivariable analysis.

RESULTS

Baseline Clinical Parameters and Pathologic Features of Enrolled Patients

Baseline demographics of the patients with DKD are shown in **Table 1**. The mean age was 55.2 years, 63.9% (53/83) were male, and the mean duration of diabetes was 11.3 years. Most patients exhibited moderate-to-severe renal dysfunction, with a mean eGFR of 45.5 mL/min/1.73 m^2 and a mean urinary protein-to-creatinine ratio of 6.0 g/gCr. During the 2.6 years of mean follow-up period, death-censored renal outcomes occurred in 35 (42.2%) of the patients. Healthy controls were significantly younger, whereas patients with combined NDRD and DKD were older, compared to those with DKD alone (p<0.001 and p=0.020, respectively; **Supplementary Table 1**). Baseline renal function and the amount of proteinuria were comparable between patients with DKD and those with combined NDRD and DKD.

Histologic examination revealed that 75.9% (63/83) of patients with DKD showed advanced glomerular injuries (36 [43.4%] and 27 [32.5%] for glomerulonephritis class III and IV, respectively; **Table 2**). Advanced tubulointerstitial fibrosis was observed in 30.1% of these patients (19 [22.9%] and 6 [7.2%] for IFTA scores of 2 and 3, respectively). Interstitial inflammation was also frequently observed, either in relation to IFTA or in areas without IFTA (44 [53.0%] and 27 [32.5%] for interstitial inflammation score of 1 and 2, respectively).

Identification of Diabetic Kidney Disease-Specific mRNA Candidates Using GEO Database

From the GEO database, we found two datasets that contained transcriptomic profiles of kidney tissues obtained from 14 DKD patients and 36 healthy kidney donors. A meta-analysis was

TABLE 1 | Baseline characteristics and clinical parameters of patients with diabetic kidney disease.

Number of patients	83
Age (year)	55.4±10.6
Sex (Male, %)	53 (63.9)
Body mass index (kg/m^2)	25.1±3.0
Duration of diabetes (years)	11.3±8.1
Presence of diabetic retinopathy (n, %)	59/80 (71.7)[a]
Hypertension (n, %)	67 (80.7)
HbA1c (%)	7.9±2.0
Hemoglobin (g/dL)	10.7±2.1
eGFR (ml/min/1.73m^2)	45.5±30.3
Albumin (g/dL)	3.2±0.6
Urine protein-to-creatinine ratio (g/gCr)	6.0±4.2
Death-censored ESKD progression (n, %)	35 (42.2)

Values are expressed as mean ± standard deviation or number of patients (percentage).
[a]Not assessed in three patients.
eGFR, estimated glomerular filtration rate; ESKD, end stage kidney disease.

TABLE 2 | Pathologic classifications of patients with diabetic kidney disease.

Glomerular classification	
Class II	20 (24.1)
Class III	36 (43.4)
Class IV	27 (32.5)
IFTA	
0	4 (4.8)
1	54 (65.1)
2	19 (22.9)
3	6 (7.2)
Interstitial inflammation	
0	12 (14.5)
1	44 (53.0)
2	27 (32.5)
Arterial hyalinosis	
0	9 (10.8)
1	56 (67.5)
2	18 (21.7)
arteriosclerosis	
0	16 (19.3)
1	51 (61.4)
2	16 (19.3)

IFTA, interstitial inflammation and tubular atrophy.

performed using the two datasets to find the relevant genes in which the expression patterns were significantly different between the groups. Among 150 genes with the lowest false discovery rate, we selected the top 20 up-regulated and 10 down-regulated genes in DKD tissues in the order of the fold changes (**Table 3**).

Urinary Levels of DKD-Specific mRNA Candidates in Different Diagnostic Groups

We next measured the levels of each mRNA candidate in the urine of healthy controls, patients with combined DKD and NDRD, and those with DKD alone. Five mRNAs failed to pass the quality control process (i.e., undetectable mRNA levels in >20% of samples) and were excluded from the analysis. Among the 17 up-regulated and 8 down-regulated mRNA candidates, 13 (76.5%) and 4 (50.0%) genes showed significantly altered expressions in the urine of patients with DKD compared to those of healthy controls, respectively (**Figure 2**). Most DKD-specific mRNA candidates up-regulated in GEO profiling were actually increased (84.6%, 11/13). In contrast, 75% of mRNAs (3/4) down-regulated in GEO profiling were paradoxically increased in the urine of patients with DKD. Notably, the expression profiles of the urinary mRNAs in patients with combined DKD and NDRD were substantially similar to those in patients with DKD alone.

TABLE 3 | List of diabetic kidney disease-specific urinary mRNA candidates identified by GEO dataset analysis.

Upregulated in DKD		Down-regulated in DKD	
Genes	**Fold change**	**Genes**	**Fold change**
LYZ	6.55	*APOLD1*	0.38
CX3CR1	4.71	*FABP1*	0.36
WFDC2	4.21	*HPD*	0.36
NNMT	4.01	*CTSV*	0.36
C3	3.72	*LPL*	0.32
MEST	3.57	*G6PC*	0.29
THBS2	3.39	*FKBP5*	0.27
MOXD1	3.09	*ZBTB16*	0.27
CLU	2.90	*PDK4*	0.23
HOPX	2.87	*CYP27B1*	0.22
COL3A1	2.86		
PLK2	2.84		
EVI2A	2.75		
TNFAIP8	2.65		
LY96	2.62		
COMP	2.51		
SPON2	2.49		
CFB	2.47		
SOX4	2.41		
COL1A1	2.39		

DKD, diabetic kidney disease.

Levels of Urinary mRNAs According to Pathologic Classification of Diabetic Kidney Disease

Subsequently, we examined the relationship between DKD-specific mRNAs and pathologic classification of DKD (**Figure 3**). Patients with glomerulonephritis class IV showed significantly higher urinary levels of five mRNAs (nicotinamide N-methyltransferase [*NNMT*], thrombospondin 2 [*THBS2*], collagen type III alpha 1 chain [*COL3A1*], spondin 2 [*SPON2*], and collagen type I alpha 1 chain [*COL1A1*]), compared with those exhibiting glomerulonephritis class II (**Figure 3A**). Meanwhile, three mRNAs (lysozyme [*LYZ*], complement 3 [*C3*], and FK506 binding protein 5 [*FKBP5*]) were positively associated with the IFTA score, while one mRNA (glucose-6-phosphatase [*G6PC*]) was negatively associated with the degree of interstitial inflammation (**Figures 3B, C**). No mRNA showed a significant relationship with the severity of arterial hyalinosis and arteriosclerosis.

Renal Outcomes According to the Clinicopathologic Features

Figure 4 shows the unadjusted Kaplan–Meier survival curves of the patients according to the stages of chronic kidney disease (CKD), amount of proteinuria, and the five different pathologic classifications. Advanced CKD stages were significantly associated with increased risks of ESKD progression, and the patients exhibiting nephrotic range proteinuria showed a trend for worse renal outcomes compared with those exhibiting non-nephrotic range proteinuria (**Figures 4A, B**). We also observed that glomerulonephritis classification, IFTA, and interstitial fibrosis were significantly associated with adverse renal outcomes (**Figures 4C–E**). Arterial hyalinosis and arteriolosclerosis were not predictive of ESKD progression (**Figures 4F, G**).

Renal Outcomes According to the Levels of Compartmental mRNA Signatures

Finally, we investigated whether urinary mRNAs can be used as the predictor of renal outcomes in patients with DKD. To this end, mRNAs associated with glomerular and tubulointerstitial injuries were integrated to generate gene signatures of each compartment. The cumulative incidence of renal outcomes was

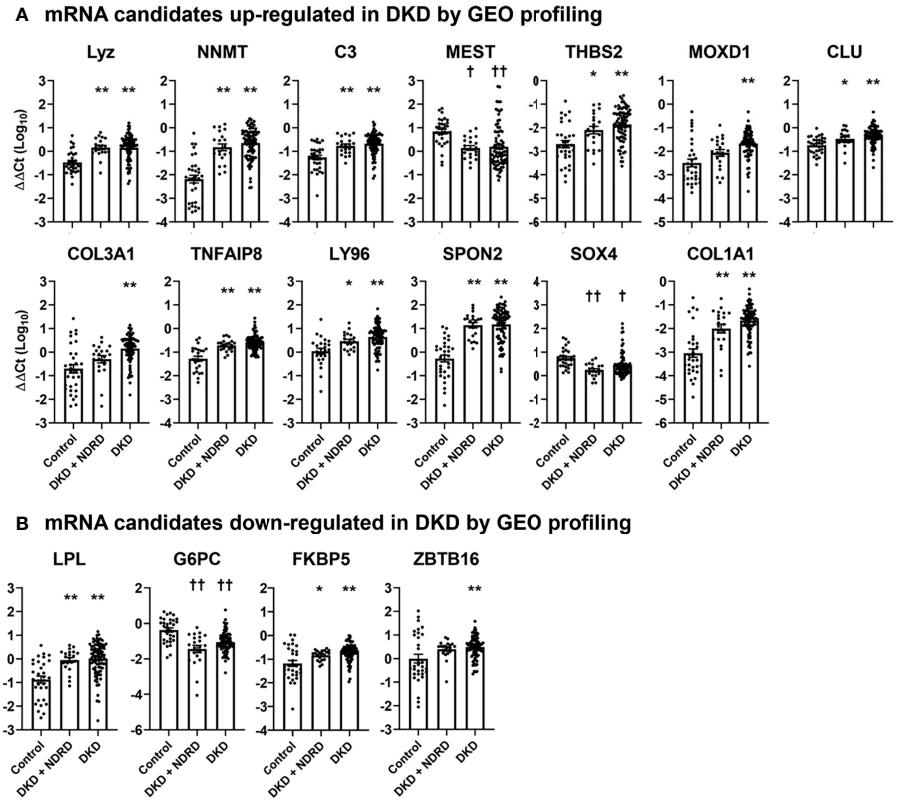

FIGURE 2 | Urinary levels of diabetic kidney disease-specific mRNA candidates in healthy controls, patients with combined diabetic kidney disease and non-diabetic renal disease, and those with isolated diabetic kidney disease. The levels of selected mRNA biomarker candidates whose expressions are significantly altered between the different groups are shown. **(A, B)** mRNA candidates up-regulated **(A)** and down-regulated **(B)** in DKD *via* GEO profiling. mRNA levels are measured by quantitative real-time polymerase chain reaction and are expressed as log-transformed delta-delta cycle threshold (ΔΔCt) after an adjustment by 18S rRNA and controls. Five mRNAs among those listed in (*CX3CR1, HOPX, COMP, APOLD1*, and *CYP27B*) are not illustrated in this figure as these mRNAs failed to pass the quality control process. $^{*}p < 0.05$, $^{**}p < 0.005$, up-regulated *vs*. control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.005$, down-regulated *vs*. control. DKD, diabetic kidney diseases; NDRD, non-diabetic renal disease; GEO, gene expression omnibus.

significantly increased in patients with third tertiles of glomerular or tubulointerstitial mRNA signatures ($p<0.001$ for both comparisons; **Figure 5**). Univariate Cox regression analysis consistently demonstrated that patients in the third tertiles of glomerular and tubulointerstitial mRNA signatures showed significantly higher risk of ESKD progression than those in the first tertiles (**Table 4**). Interestingly, the significant associations between glomerular mRNA signatures and renal outcomes disappeared when baseline renal function was added as an adjustment variable (hazard ratios [HR] of 1.80, 95% confidence interval [CI] of 0.46–7.06, $p=0.402$). In contrast, tubulointerstitial mRNA signatures maintained their significant associations with poor renal outcomes even after the adjustments with baseline renal function (HR of 9.68, 95% CI of 2.85–32.87, $p<0.001$).

DISCUSSION

In this study, we analyzed the clinicopathologic data and various urinary mRNAs to discover novel, non-invasive biomarkers that could predict renal outcomes in patients with biopsy-proven DKD. Utilizing public GEO datasets, we extracted 30 mRNAs as biomarker candidates; we observed that levels of 17 mRNAs were significantly altered in the urine of patients with DKD, compared to those of healthy controls. Among these, five and four mRNAs showed significant associations with the pathologic severity of glomerular and tubulointerstitial compartments, respectively. Finally, four urinary mRNAs—*LYZ, C3, FKBP5*, and *G6PC*—were observed to be associated with tubulointerstitial injury and could predict DKD progression independently from baseline clinical parameters, including residual kidney functions. Together, these data suggest that urinary tubulointerstitial mRNA signatures may help identify those at high risk of progression to ESKD.

Urine is a valuable source for identifying relevant biomarkers associated with kidney diseases as it is generated directly from the kidneys and can be collected non-invasively. We have previously demonstrated the utility of urinary mRNAs and proteins as diagnostic and prognostic biomarkers in various renal conditions such as transplant rejection, primary glomerular diseases, and DKD (18, 22–28). Recent

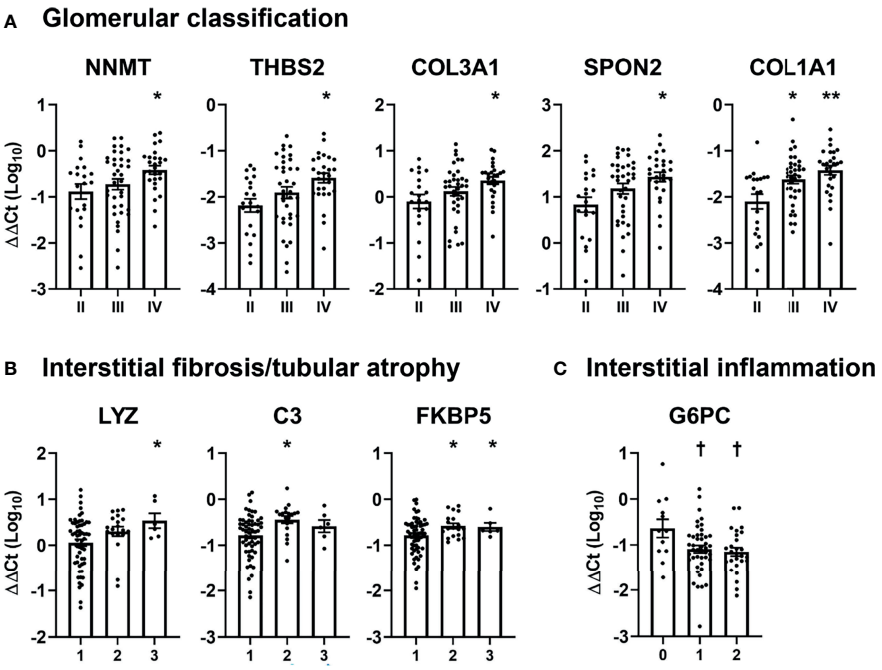

FIGURE 3 | Association between pathologic classifications and urinary mRNA levels in patients with diabetic kidney disease. **(A–C)** The levels of significantly altered urinary mRNAs according to **(A)** glomerular classification, **(B)** interstitial fibrosis and tubular atrophy, and **(C)** interstitial inflammation scores in patients with diabetic kidney disease are shown. Levels of each mRNA are expressed as log-transformed delta-delta cycle threshold (ΔΔCt) after adjusting for 18S rRNA and controls. $^{*}p < 0.05$, $^{**}p < 0.005$, up-regulated *vs*. glomerular class II or IFTA score of 1; $^{\dagger}p < 0.05$, down-regulated *vs*. interstitial inflammation score of 0.

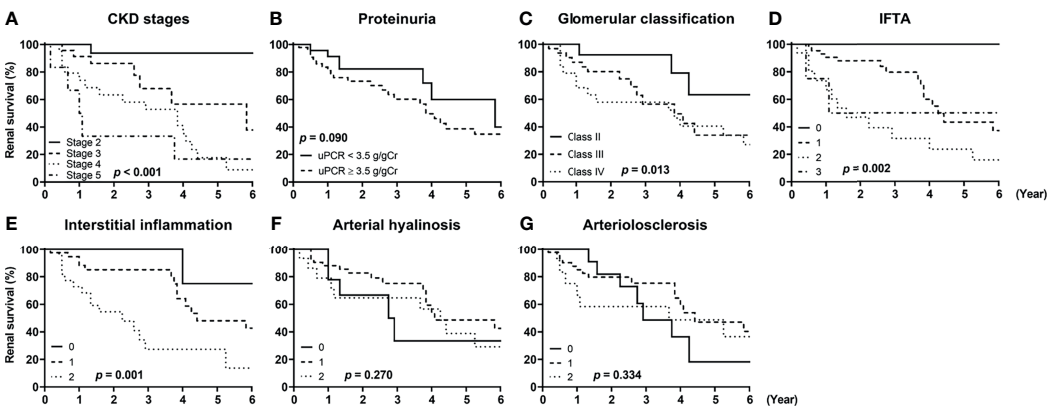

FIGURE 4 | Renal survival of patients with diabetic kidney disease according to their clinicopathologic features. The renal survival of patients with diabetic kidney disease according to **(A)** stages of chronic kidney disease (CKD), **(B)** the amount of proteinuria, **(C)** glomerulonephritis classification score, **(D)** interstitial fibrosis and tubular atrophy (IFTA) score, **(E)** interstitial inflammation score, **(F)** arterial hyalinosis score, and **(G)** arteriolosclerosis score are shown. *P*-values were calculated by log-rank test. CKD, chronic kidney disease; uPCR, urinary protein-to-creatinine ratio; IFTA, interstitial fibrosis and tubular atrophy.

advances in the utilization of open data resources have further enhanced the potentials of urinary mRNAs in identifying biomarkers. Using open datasets of DKD and applying an integrative bioinformatics approach, Zhou et al. revealed urinary *BBOX1* to be a non-invasive diagnostic biomarker of DKD in diabetic patients who did not undergo kidney biopsy (29). In this study, we were able to eliminate the possibility of the presence of unexpected NDRD and determine the relationship between renal histology and urinary mRNAs by including patients whose diagnosis was confirmed by renal biopsy, emphasizing the importance of pathologic data in a DKD study.

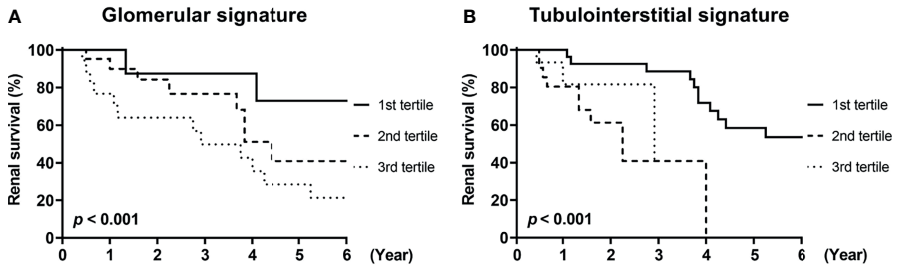

FIGURE 5 | Renal survival of patients with diabetic kidney disease according to compartmental mRNA signatures. **(A, B)** The renal survival of patients with diabetic kidney disease according to the tertiles of **(A)** glomerular and **(B)** tubulointerstitial mRNA signatures are shown. Each signature was generated from the integration of mRNAs differentially expressed in corresponding compartments (*NNMT, THBS2, SPON2, COL3A1, COL1A1* for glomerular signature and *LYZ, C3, FKBP5, G6PC* for tubulointerstitial signature). $p < 0.001$ for both comparisons by log-rank test.

TABLE 4 | Hazard ratios of compartmental mRNA signatures for renal survival.

		Unadjusted		Model 1[a]		Model 2[b]	
		HR (95% CI)	***p* value**	**HR (95% CI)**	***p* value**	**HR (95% CI)**	***p* value**
Glomerular signatures[c]	Tertile 1	Reference	–	Reference	–	Reference	–
	Tertile 2	2.62 (0.82 – 8.40)	0.106	2.82 (0.84 – 9.47)	0.093	1.61 (0.43 – 5.96)	0.477
	Tertile 3	6.50 (2.22 – 19.08)	0.001	6.07 (1.93 – 19.06)	0.002	1.80 (0.46 – 7.06)	0.402
Tubulointerstitial signatures[d]	Tertile 1	Reference	–	Reference	–	Reference	–
	Tertile 2	6.93 (2.35 – 20.40)	<0.001	11.47 (3.27 – 40.24)	<0.001	7.77 (2.51 – 23.68)	<0.001
	Tertile 3	7.62 (2.24 – 25.92)	0.001	11.73 (3.07 – 44.87)	<0.001	9.68 (2.85 – 32.87)	<0.001

[a]*Model 1: adjusted for age, sex, hypertension, and urinary protein-to-creatinine rate.*
[b]*Model 2: model 1 + estimated glomerular filtration rate.*
[c]*Composed of urinary NNMT, THBS2, SPON2, COL3A1, and COL1A1 mRNA levels.*
[d]*Composed of urinary FKBP5, C3, LYZ, and G6PC mRNA levels.*
HR, hazard ratio; CI, confidence interval.

The clinical significance of pathologic classifications of DKD in predicting renal outcomes has been consistently demonstrated in previous studies (14–18), supporting the idea that urinary mRNAs reflecting intrarenal pathology could be prognostic biomarkers in patients with DKD. In this study, we observed that several DKD-specific urinary mRNAs were significantly associated with the severity of pathologic findings in the kidneys as well as renal outcomes (**Figures 3** and **5**). Although the pathophysiologic roles of selected mRNAs were not investigated here, previous studies have shown glomerular compartmental mRNAs, comprising *NNMT, THBS2, SPON2, COL3A1*, and *COL1A1*, to be involved in podocyte damage (30–32) and glomerulosclerosis (33, 34), and tubulointerstitial compartmental mRNAs, comprising *LYZ, C3, FKBP5*, and *G6PC*, to be associated with fibrosis (35–37) and inflammation (38). Notably, those mRNAs reflected different compartments of the kidneys in an exclusive manner, suggesting that glomerular and tubulointerstitial injuries might result in discriminative urinary mRNA expressions. In line with our data, a recent study performed transcriptomic analysis of micro-dissected kidneys and showed discriminative gene expression patterns between glomerular and tubulointerstitial compartments (39).

Among the differentially expressed mRNAs, those up-regulated in the patients with DKD were predominantly involved in immune response and inflammation (*CLU, C3, CFB, LY96, SPON2, CX3CR1, FKBP5, TNFAIP8*), and extracellular matrix organization (*COMP, COL1A1, COL3A1, THBS2, SPON2, MOXD1*); those down-regulated in the patients with DKD were mainly associated with metabolic pathways (*APOLD1, FABP1, HPD, LPL, G6PC, PDK4*). The overall trends were consistent with those reported in previous studies that have investigated transcriptomic profiles of renal tissues obtained from advanced human diabetic nephropathy (39, 40). Notably, most mRNAs (11/13, 84.6%) among those up-regulated in patients with DKD *via* GEO profiling showed increased levels in the urine. In contrast, only one mRNA (1/4, 25%) among those down-regulated in patients with DKD *via* GEO profiling showed decreased levels in the urine (**Figures 2A** and **1B**). Although the reasons for this discrepancy could not be identified in this study, the mRNA expression profiles of the cells might have been altered once they were detached from the kidneys and released into the urine.

Our data suggest that urinary mRNAs may be potential predictors of renal function decline in patients with advanced DKD. In particular, mRNA signatures of tubulointerstitial inflammation and fibrosis were a significant predictor of poor renal outcomes even after multivariable adjustments, including baseline renal function. In contrast, the predictive power of glomerular mRNA signatures in predicting renal outcomes was lost after adjustments for eGFR. These results suggest that

tubulointerstitial mRNA signatures may be potential independent predictors of rapid decline in renal function, whereas glomerular mRNA signatures are not. Similarly, in line with the findings of previous studies, we revealed the advantages of tubulointerstitial injury scores over glomerular classifications in the prediction of renal outcomes among patients with DKD exhibiting advanced glomerular injuries (16, 18).

Normalization of urinary mRNA expression data is a critical issue in biomarker research; however, optimal normalization strategies for mRNA remain controversial (41). In this study, we used 18S rRNA rather than urine creatinine for the normalization of urinary mRNAs expression data as we have previously demonstrated this strategy to be useful in identifying urinary mRNA biomarkers (22, 28). Further investigations are required to determine whether urine creatinine may be better for normalization of urinary mRNA expression data.

The limitations of this study should be mentioned. We did not determine whether the mRNA signatures developed in this study could be applied to patients with early-stage DKD. Patients with early-stage DKD were not included in this study as they rarely undergo renal biopsy in clinical practice. Given that early and advanced diabetic nephropathy shows substantially different transcriptomic profiles (40), biomarkers of advanced DKD may not be useful in the early stages of DKD. In addition, DKD-specific urinary mRNA profiles could not discriminate between patients with DKD and those with combined DKD and NDRD. A possible reason for this may be that the patients in both groups had a substantial duration of diabetes (mean duration >10 years); therefore, the effects of NDRD on urinary mRNA levels were relatively insignificant compared to those of DKD. The smaller number of patients in the NDRD group as well as their diagnostic heterogeneity might have also affected these results.

In conclusion, we developed urinary mRNA signatures as predictors of rapid disease progression in patients with advanced DKD. Future prospective studies are required to confirm whether our mRNA signatures can identify those at high risk of renal function decline in a non-invasive manner.

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 IRB no. KHNMC2021-01-054-003. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Research idea and study design: YL, J-WS, and J-YM. Data acquisition: YK, S-HL, JSK, HH, and K-HJ. Data analysis/interpretation: YL, J-WS, DT, and JSK. Statistical analysis: YL, J-WS, DT, and JSK. Supervision or mentorship: J-YM. Each author contributed important intellectual content during manuscript drafting and approved the final article.

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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.2021.774436/full#supplementary-material

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The Risk of Nephropathy, Retinopathy, and Leg Amputation in Patients With Diabetes and Hypertension: A Nationwide, Population-Based Retrospective Cohort Study

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Purpose: To compare the risks of chronic kidney disease (CKD), end-stage renal disease (ESRD), sight-threatening retinopathy, and leg amputation between patients with diabetes or hypertension.

Methods: From January 1, 2000, to December 31, 2015, we identified 28943 matched pairs of patients with diabetes with and without subsequent hypertension, 89102 pairs of patients with hypertension with and without subsequent diabetes, and 145294 pairs of patients with coexisting diabetes and hypertension with a previous history of diabetes or hypertension from Taiwan's National Health Insurance Research Database. Cox proportional-hazard models were used for calculating the risks of CKD, sight-threatening retinopathy, and leg amputation.

Results: The mean follow-up time of this study in different cohorts was between 3.59 and 4.28 years. In diabetes patients with vs. without subsequent hypertension, hypertension patients with vs. without subsequent diabetes, and comorbid diabetes and hypertension patients with previous diabetes vs. with previous hypertension, the adjusted HRs (95% CIs) for CKD were 2.77 (2.61-2.94), 1.73 (1.68-1.77), and 1.04 (1.02-1.07); for ESRD were 42.38 (22.62-79.4), 2.76 (2.43-3.13), and 0.72 (0.66-0.79); for sight-threatening retinopathy were 2.07 (1.85-2.3), 3.41 (3.14-3.71), and for leg amputation were 1.51 (1.43-1.58); and 4.74 (3.02-7.43), 6.27(4.72-8.31), and 1.19(1.03-1.38).

Conclusions: This study demonstrated that both diabetes and hypertension are risk factors for the development of CKD, retinopathy, and amputation. Tracing subsequent

diabetes for patients with hypertension, and hypertension for patients with diabetes are important in clinical settings.

Keywords: chronic kidney disease, end-stage renal disease, sight-threatening retinopathy, leg amputation, diabetes and hypertension

INTRODUCTION

Hypertension is one of the most common chronic diseases in the world (1). It can lead to cardiovascular diseases and chronic kidney disease (2). High systolic blood pressure is the leading risk factor for attributable deaths, accounting for 10.8 million deaths worldwide and 19.2% of all deaths in 2019 (3). Type 2 diabetes mellitus, one of the leading chronic diseases globally, is linked to lifestyle factors. In 1990, approximately 148 million people worldwide had diabetes, and the number tripled to about 438 million in 2019. The prevalence rate also increased from 2.88% in 1990 to 5.89% in 2019 (4). Patients with diabetes are prone to developing macrovascular and microvascular complications, which increase the risk of mortality.

Chronic kidney disease is a silent deterioration of renal function to estimated glomerular filtration rate (eGFR) less than 60 ml/min/1.73m^2 or abnormal markers of renal damage for more than 3 months (5). If unmanaged, it may progress to end-stage renal disease (ESRD) and increase the risk of cardiovascular disease and premature death (5). Retinopathy involves abnormal changes in the small retinal blood vessels. It is the leading cause of blindness among working-age adults (6). Poor blood circulation in the distal limbs can lead to leg amputation, the last resort in managing poorly healing wounds that worsens the quality of life in patients (7).

Reports suggest that hypertension and diabetes are the main risk factors for CKD, retinopathy, and amputation (5, 8, 9). Diabetes is the most common cause of ESRD; up to 80% of ESRD is caused by diabetes, hypertension, or a combination of both (10). Population aging in Taiwan has resulted in an increasing prevalence of diabetes and CKD in recent years. Taiwan has the highest incidence and prevalence of dialysis in the world (11). Because few studies have investigated the different impacts of diabetes and hypertension on microvascular complications, we conducted this study to compare whether diabetes with or without subsequent hypertension, hypertension with or without subsequent diabetes, and coexisting hypertension and diabetes with a previous history of hypertension or diabetes, differ in their impacts on the risks of CKD, ESRD, sight-threatening retinopathy, and leg amputation.

MATERIALS AND METHODS

Study Population

We identified patients with newly diagnosed type 2 diabetes mellitus or hypertension from the National Health Insurance Research Database (NHIRD) between January 1, 2000, and December 31, 2015. The NHIRD contains medical records of National Health Insurance (NHI) from 1995 to the present (12). It includes information on patient sex, age, place of residence, procedure, therapy, and diagnosis according to the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM), and ICD-10-CM codes. It involves the health services of inpatient admissions, outpatient visits, and emergency department visits. The NHIRD is linked to the National Death Registry to certify mortality information. The NHI program was implemented by the Taiwan government in 1995. It is a compulsory insurance system, with most of the premiums paid by the government and employers. By 2000, approximately 99% of the 23 million people in Taiwan were insured. Our study was approved by the Research Ethics Committee of China Medical University and Hospital (CMUH109-109-REC2-031). The identifier information of patients or care providers was di-identified and encrypted before release to protect individual privacy. Therefore, informed consent from patients was not required.

Study Design

Type 2 diabetes mellitus and hypertension were diagnosed by ICD-9-CM codes or ICD-10-CM codes (**Supplementary Table 1**), with at least 2 outpatient claims within 1 year or one hospitalization. This algorithm of using ICD codes has been validated by previous studies with the accuracy of diabetes was 74.6% (13), the sensitivity and specificity of hypertension were 92.4% and 59.9% (14). We excluded patients diagnosed with type 1 diabetes, younger than 20 years or older than 80 years (patients with too old age could have poor renal function or frail syndromes which may interfere with the results), lacking age or gender information, diagnosed with chronic kidney disease (CKD), having dialysis, retinopathy, visual loss, leg amputation, heart failure, and hepatic failure before the index date. We also excluded patients who died or were followed-up for less than 180 days after the index date (to avoid confounding effects of the latent morbidities).

Procedures

In this retrospective cohort study, we constructed 3 cohorts from 2000 to 2015 to compare the risks of CKD, retinopathy, and amputation in relation to diabetes and hypertension (**Figure 1**): (a) diabetes patients with and without subsequent hypertension (diabetes cohort), (b) hypertension patients with and without subsequent diabetes (hypertension cohort), (c) patients with coexisting diabetes and hypertension (comorbid cohort). The coexisting diabetes and hypertension indicates a patient has at least 2 outpatient claims within 1 year or one hospitalization due to both diseases. For the diabetes cohort, of 181018 newly

Abbreviations: CKD, chronic kidney disease; ESRD, end-stage renal disease; CCI, Charlson Comorbidity Index; DCSI, Diabetes Complication Severity Index.

diagnosed diabetes patients, after excluding ineligible patients, there were 103289 patients with subsequent hypertension and 77729 patients without subsequent hypertension. We defined the date of first hypertension diagnosis as the index date of this diabetes cohort. For the hypertension cohort, of 51224 newly diagnosed hypertension patients, after excluding ineligible patients, there were 160243 patients with subsequent diabetes and 360981 patients without subsequent diabetes. We defined the date of subsequent diabetes diagnosis as the index date of this hypertension cohort. For the comorbid cohort, of 643830 patients with coexisting diabetes and hypertension, after excluding ineligible patients, there were 416825 patients with previous diabetes and 227005 patients with previous hypertension. We defined the date of concurrent diabetes and hypertension diagnosis as the index date of this comorbid cohort. Within each cohort, we assigned the same index date for the two comparison subgroups to avoid immortal time bias. The detailed study designs were delineated in **Supplementary Table 2**.

Variables considered as potential confounders in this study were as follows: sex, age, overweight, obesity, severe obesity, smoking, dyslipidemia, coronary artery disease (CAD), stroke, atrial fibrillation, chronic obstructive pulmonary disease (COPD), liver cirrhosis, peripheral arterial occlusion disease (PAOD); Charlson Comorbidity Index (CCI) (15) and Diabetes Complication Severity Index (DCSI) scores (16); number and item of oral antidiabetic medications and insulin (**Table 1**); number and item of antihypertensive medications (**Table 2**); non-steroidal anti-inflammatory drugs (NSAIDs); statin; aspirin (**Tables 2**, **3**); duration of diabetes (the duration of first diabetes diagnosis to the index date. **Table 1**); duration of hypertension (the duration of first hypertension diagnosis to the index date. **Table 2**).

Main Outcomes

We investigated the development of the following conditions: CKD, end-stage renal disease (ESRD) defined as patients receiving dialysis, sight-threatening retinopathy defined as patients with at least two outpatient visits or one admission for retinopathy requiring surgery or receiving laser photocoagulation within 90 days of retinopathy diagnosis, or with visual loss, or receiving anti-vascular endothelial growth factor injection (ranibizumab, bevacizumab, or aflibercept); leg amputation defined by the ICD coding in at least one hospitalization. The incidence rates of CKD, ESRD, sight-threatening retinopathy, and leg amputation were calculated and compared between the comparison subgroups within each study cohort.

Statistical Analysis

Propensity score matching was used to optimize comparability between the comparison subgroups within each study cohort (17). The propensity score for every patient was estimated using non-parsimonious multivariable logistic regression. Approximately 20 clinically related variables were used in the

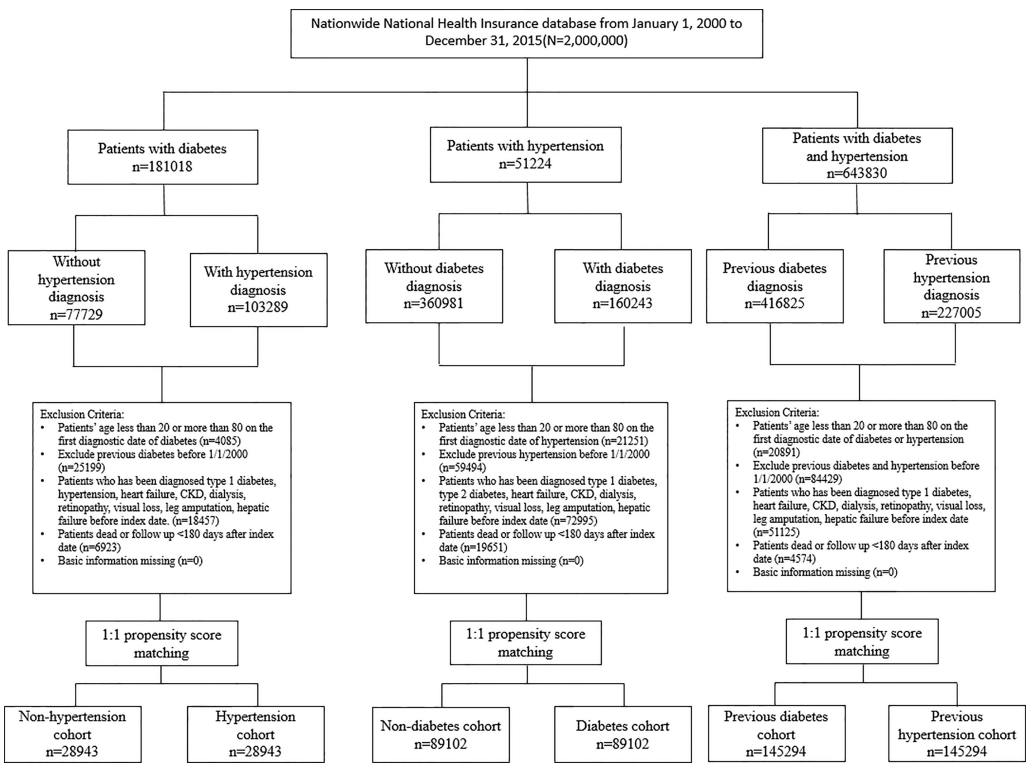

FIGURE 1 | Flow chart of the identified study population.

TABLE 1 | Comparison of baseline characteristics of the study subjects in the diabetes cohort.

Variables	Without subsequent hypertension (N = 28943)		With subsequent hypertension (N = 28943)		SMD
	n	%	n	%	
Sex					
Female	13464	46.52	13454	46.48	0.001
Male	15479	53.48	15489	53.52	0.001
Age					
20-39	3920	13.54	3939	13.61	0.002
40-59	17477	60.38	17450	60.29	0.002
60-80	7546	26.07	7554	26.10	0.001
Mean, (SD)	53.2	11.48	53.21	11.51	0.001
Comorbidities					
Obesity					
Overweight	560	1.93	564	1.95	0.001
Normal Obesity	451	1.56	441	1.52	0.003
Severe obesity	53	0.18	59	0.20	0.005
Smoking	588	2.03	618	2.14	0.007
Dyslipidemia	15816	54.65	16107	55.65	0.02
Coronary artery disease	3897	13.46	3872	13.38	0.003
Stroke	1197	4.14	1152	3.98	0.008
Atrial fibrillation	11	0.04	16	0.06	0.008
PAOD	656	2.27	671	2.32	0.003
COPD	5643	19.50	5766	19.92	0.011
Liver cirrhosis	744	2.57	772	2.67	0.006
CCI					
1	9540	32.96	9289	32.09	0.019
2-3	13933	48.14	13981	48.31	0.003
>3	5470	18.90	5673	19.60	0.018
DCSI					
0	13902	48.03	13863	47.90	0.003
1	5809	20.07	5803	20.05	0.001
≥2	9232	31.90	9277	32.05	0.003
Medication					
Metformin	13849	47.85	13957	48.22	0.007
Sulfonylurea	12719	43.95	13323	46.03	0.042
TZD	2680	9.26	2774	9.58	0.011
DPP-4i	1571	5.43	1409	4.87	0.025
AGI	2718	9.39	2918	10.08	0.023
Number of OAD					
0-1	17424	60.20	17267	59.66	0.011
2-3	10046	34.71	10184	35.19	0.01
>3	1473	5.09	1492	5.16	0.003
Insulin	10369	35.83	10377	35.85	0.001
Statin	8082	27.92	8270	28.57	0.02
NSAIDs	28139	97.22	28231	97.54	0.014
Diabetes duration, (SD)	3.69	3.33	3.59	3.51	0.03

SMD, standardized mean difference. A standardized mean difference of 0.05 or less indicates a negligible difference.
PAOD, peripheral arterial occlusive disease; COPD, chronic obstructive pulmonary disease; CCI, Charlson comorbidity index; DCSI, diabetes complication severity index; TZD, thiazolidinedione; DPP-4i, Dipeptidyl peptidase-4 inhibitor; AGI, Alpha-glucosidase inhibitors; NSAIDs, non-steroidal anti-inflammatory drugs; OAD, oral anti-diabetic drugs.

analysis as controlling variables (**Tables 1–3**). A standardized mean difference (SMD) algorithm was utilized to construct matching pairs under the assumption that a standardized mean difference of 0.05 or less indicated a negligible difference.

The incidence rates for each outcome were measured by the number of cases per 1,000 person-years. The person-years were calculated as the time from the index date to the date of the event, death, or the end of follow-up (December 31st, 2015), whichever came first. Crude and multivariate-adjusted Cox proportional hazard models were employed to compare the risk of outcomes between the study and comparison groups. The proportional hazards assumption was not violated by comparing estimated log-log survival curves for all time independent covariates. The results were presented as hazard ratios (HRs) and 95% confidence intervals (CIs) for study versus comparison groups. Because the competing risks of death might confound the estimates of risks for our investigated outcomes, we applied the Fine and Gray's sub-distribution hazard model for adjustment. To assess risk for each investigated outcome, we censored patients on the date of death, the date of respective outcomes, or end of follow-up on 31 December 2015, whichever occurred first. A two-tailed *P* value less than 0.05 was considered

TABLE 2 | Comparison of baseline characteristics of the study subjects in the hypertension cohort.

Variables	Without subsequent diabetes (N = 89102)		With subsequent diabetes (N = 89102)		SMD
	n	%	n	%	
Sex					
Female	43646	48.98	44382	49.8	0.017
Male	45456	51.02	44720	50.2	0.017
Age					
20-39	5613	6.30	5604	6.3	0
40-59	44125	49.52	44279	49.7	0.003
60-80	39364	44.18	39219	44.0	0.003
mean, (SD)	58.58	11.50	58.55	11.5	0.003
Obesity					
Overweight	1055	1.18	1174	1.3	0.012
Normal Obesity	831	0.93	917	1.0	0.01
Severe obesity	122	0.14	160	0.2	0.011
Smoking status	1074	1.21	1274	1.4	0.02
Comorbidities					
Dyslipidemia	36784	41.28	38830	43.6	0.046
Coronary artery disease	24956	28.01	26648	29.9	0.042
Stroke	20	0.02	13	0.0	0.006
Atrial fibrillation					
PAOD	2217	2.49	2569	2.9	0.024
COPD	21069	23.65	22328	25.1	0.033
Liver cirrhosis	1147	1.29	1402	1.6	0.024
CCI					
1	28099	31.54	26612	29.9	0.036
2-3	43709	49.06	43636	49.0	0.002
>3	17294	19.41	18854	21.2	0.044
Medication					
ACEI/ARB	30382	34.10	31026	34.8	0.015
β-blockers	59179	66.42	59203	66.4	0.001
Calcium-channel blockers	64955	72.90	64742	72.7	0.005
Diuretics	43497	48.82	46378	52.1	0.065
Number of hypertension drugs					
1	26787	30.06	25079	28.1	0.042
2-3	46939	52.68	47825	53.7	0.02
>3	15376	17.26	16198	18.2	0.024
Statin	19498	21.88	20877	23.4	0.037
Aspirin	32775	36.78	34187	38.4	0.033
NSAIDs	84529	94.87	85543	96.0	0.055
Hypertension duration, (SD)	4.28	3.58	4.26	3.69	0.005

SMD, standardized mean difference. A standardized mean difference of 0.05 or less indicates a negligible difference.
PAOD, peripheral arterial occlusive disease; COPD, chronic obstructive pulmonary disease; CCI, Charlson comorbidity index; ACEI, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers; NSAIDs, non-steroidal anti-inflammatory drugs.

significant. SAS v9.4 (SAS Institute, Inc., Cary, NC, USA) was used for the analysis.

RESULTS

Study Population

In the diabetes cohort, after propensity score matching, 28943 pairs of matched patients were selected (**Table 1**). The mean follow-up time was 3.69 years for diabetes persons with subsequent hypertension and 3.59 years for persons without subsequent hypertension. In the hypertension cohort, 89102 pairs of matched patients were selected (**Table 2**). The mean follow-up time was 4.28 years for hypertension persons with subsequent diabetes and 4.26 years for persons without subsequent diabetes. In the cohort of coexisting diabetes and hypertension, 145294 pairs of matched patients were selected (**Table 3**). The mean follow-up time was 3.79 years for persons with previous diabetes and 4.07 years for persons with previous hypertension.

Main Outcomes

In people with diabetes, those with subsequent hypertension had substantially higher risks of CKD (aHR=2.77, 95% CI 2.61-2.94) and ESRD (aHR=42.38, 95% CI 22.62-79.4) compared to those without hypertension (**Table 4**). In patients with hypertension, those with subsequent diabetes showed prominently higher risks of sight-threatening retinopathy (aHR=3.41, 95% CI 3.14-3.71) and leg amputation (aHR=6.27, 95% CI 4.72-8.31) than those without diabetes (**Table 4**). In patients with coexisting diabetes

TABLE 3 | Comparison of baseline characteristics of the study subjects in the comorbid cohort.

Variables	With previous diabetes (N = 145294)		With previous hypertension (N = 145294)		SMD
	n	%	n	%	
Sex					
Female	72020	49.57	72937	50.20	0.013
Male	73274	50.43	72357	49.80	0.013
Age					
20-39	20128	13.85	18948	13.04	0.024
40-59	76192	52.44	75399	51.89	0.011
60-80	48974	33.71	50947	35.06	0.029
Mean, (SD)	54.69	12.73	55.18	12.75	0.038
Obesity					
Overweight	1486	1.02	1454	1.00	0.002
Obesity	1101	0.76	1097	0.76	0
Severe obesity	150	0.10	199	0.14	0.01
Smoking status	1872	1.29	1869	1.29	0
Comorbidities					
Dyslipidemia	51072	35.15	50562	34.80	0.007
Coronary artery disease	27611	19.00	28518	19.63	0.016
Stroke	8022	5.52	8451	5.82	0.013
Atrial fibrillation	971	0.67	1012	0.70	0.003
PAOD	2669	1.84	2841	1.96	0.009
COPD	29757	20.48	29649	20.41	0.002
Liver cirrhosis	2341	1.61	2224	1.53	0.006
CCI					
0	52737	36.30	53426	36.77	0.01
1	67119	46.20	65933	45.38	0.016
≥2	25438	17.51	25935	17.85	0.009
DCSI					
0	80797	55.61	81112	55.83	0.009
1	25688	17.68	26997	18.58	0.009
≥2	38809	26.71	37185	25.59	0.009
Medications					
Metformin	22843	15.72	16833	11.59	0.121
Sulfonylurea	23979	16.50	18466	12.71	0.108
TZD	4934	3.40	1406	0.97	0.167
DPP-4i	1435	0.99	424	0.29	0.087
AGI	4727	3.25	1546	1.06	0.151
Insulin	39909	27.47	37694	25.94	0.034
Number of OAD					
≦1	125118	86.11	133454	91.85	0.009
2-3	17631	12.13	11548	7.95	0.009
>3	2545	1.75	292	0.20	0.009
ACEI/ARB	9103	6.27	34867	24.00	0.511
β-blockers	47858	32.94	73983	50.92	0.371
Calcium-channel blockers	30195	20.78	73095	50.31	0.648
Diuretics	31638	21.78	55676	38.32	0.367
Number of hypertension drugs					
1	113187	77.90	72356	49.80	0.612
2-3	30476	20.98	56009	38.55	0.392
>3	1631	1.12	16929	11.65	0.441
Statin	22774	15.67	23502	16.18	0.014
Aspirin	37910	26.09	38930	26.79	0.016
NSAIDs	134184	92.35	133289	91.74	0.023
Diabetes duration					1.601
mean, (SD)	3.79	3.35	–	–	
Hypertension duration					1.621
mean, (SD)	–	–	4.07	3.55	

SMD, standardized mean difference. A standardized mean difference of 0.05 or less indicates a negligible difference.

PAOD, peripheral arterial occlusive disease; COPD, chronic obstructive pulmonary disease; CCI, Charlson comorbidity index; DCSI, Diabetes Complication Severity Index; TZD, thiazolidinedione; DPP-4i, Dipeptidyl peptidase-4 inhibitor; AGI, Alpha-glucosidase inhibitors; ACEI, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers; NSAIDs, non-steroidal anti-inflammatory drugs. OAD, oral anti-diabetic drugs.

and hypertension, those with a previous history of hypertension showed a significantly lower risk of ESRD than those with previous diabetes (aHR=0.72); patients with a history of hypertension exhibited higher risks of CKD, sight-threatening retinopathy, and leg amputation than those with a history of diabetes (aHR: 1.04, 1.51, and 1.19, respectively **Table 4**).

In brief, diabetes seemed to be an important risk factor for developing ESRD, sight-threatening retinopathy, and leg amputation; and hypertension was also an overlooked worsening factor for CKD and ESRD as shown in this study.

DISCUSSION

Our study demonstrated that (1). Among patients with diabetes, those with subsequent hypertension showed higher risks of CKD, ESRD, sight-threatening retinopathy, and leg amputation than those without subsequent hypertension. (2). Among patients with hypertension, those with subsequent diabetes demonstrated higher risks of CKD, ESRD, sight-threatening retinopathy, and leg amputation than those without subsequent diabetes. (3). Among patients with coexisting diabetes and hypertension, those with previous hypertension showed increased risks of CKD, retinopathy, and leg amputation, while those with a previous history of diabetes exhibited a higher risk of ESRD.

Approximately 10-15% of the population (18) and nearly 700 million people worldwide have CKD (5). CKD can increase the risk of cardiovascular disease and significantly shorten life expectancy (18). In 2019, approximately 1.4 million people died from CKD (4). CKD was the 12th global leading cause of death in 2017 (19). Diabetes is the main risk factor for CKD (18, 19), and estimates suggest that about 50% of persons with type 2 diabetes will develop CKD (20). A cross-sectional study in Korea revealed that patients with diabetes showed a higher risk of CKD than patients with hypertension (21). Our study also demonstrated that patients with hypertension and subsequent diabetes showed a higher risk of CKD. Hyperglycemia may produce reactive oxygen species (ROS). ROS plays a key role in mesangial matrix expansion, tubule-interstitial fibrosis, podocyte loss, and CKD development (20). Several studies have revealed that intensive glucose control in persons with diabetes can reduce the risk of incident CKD, especially in reducing proteinuria (20). However, the best way to reduce the risk of incident CKD may be to prevent the occurrence of diabetes. Patients with hypertension should reduce the intake of sugar-sweetened beverages, control obesity, and increase physical activity to reduce the incidence of diabetes and mitigate CKD risk.

In 2010, approximately 31.1% of adults (1.39 billion) worldwide had hypertension (22). Hypertension is an important risk factor for CKD development and progression (18, 19). CKD can multiply the risk of cardiovascular death in patients with diabetes and hypertension (19). The study by the

TABLE 4 | HRs and 95% CIs for the outcomes of CKD, ESRD, retinopathy, and amputation.

Outcome	Diabetes persons						cHR	(95% CI)	p-value	aHR[a]	(95% CI)	p-value
	Without subsequent hypertension (n = 28943)			With subsequent hypertension (n = 28943)								
	n	PY	IR	n	PY	IR						
CKD	1438	214511	6.7	3837	207142	18.52	2.78	(2.62, 2.96)	<0.001	2.77	(2.61, 2.94)	<0.001
ESRD	10	219012	0.05	407	220305	1.85	40.8	(21.8, 76.41)	<0.001	42.38	(22.62, 79.4)	<0.001
Sight-threatening retinopathy	483	216802	2.23	998	216496	4.61	2.07	(1.85, 2.3)	<0.001	2.07	(1.85, 2.3)	<0.001
Leg amputation	23	218983	0.11	111	221107	0.5	4.78	(3.05, 7.5)	<0.001	4.74	(3.02, 7.43)	<0.001
Outcome	**Hypertension persons**						**cHR**	**(95% CI)**	**p-value**	**aHR**	**(95% CI)**	**p-value**
	Without subsequent diabetes (n=89102)			With subsequent diabetes (n=89102)								
	n	PY	IR	n	PY	IR						
CKD	9179	781780	11.74	15028	751578	20	1.73	(1.68, 1.77)	<0.001	1.73	(1.68, 1.77)	<0.001
ESRD	332	819586	0.41	890	812346	1.1	2.74	(2.42, 3.11)	<0.001	2.76	(2.43, 3.13)	<0.001
Sight-threatening retinopathy	722	816799	0.88	2380	799469	2.98	3.37	(3.1, 3.66)	<0.001	3.41	(3.14, 3.71)	<0.001
Leg amputation	56	820498	0.07	348	813669	0.43	6.3	(4.75, 8.35)	<0.001	6.27	(4.72, 8.31)	<0.001
Outcome	**Coexisted diabetes and hypertension persons**						**cHR**	**(95% CI)**	**p-value**	**aHR**	**(95% CI)**	**p-value**
	Previous diabetes history (n=145294)			Previous hypertension history (n=145294)								
	n	PY	IR	n	PY	IR						
CKD	17591	1338198	13.15	20497	1309776	15.65	1.2	(1.17, 1.22)	<0.001	1.04	(1.02, 1.07)	<0.001
ESRD	1382	1414080	0.98	1221	1395614	0.87	0.91	(0.84, 0.98)	0.01	0.72	(0.66, 0.79)	<0.001
Sight-threatening retinopathy	3242	1397556	2.32	4380	1369825	3.2	1.38	(1.31, 1.44)	<0.001	1.51	(1.43, 1.58)	<0.001
Leg amputation	413	1417057	0.29	520	1397342	0.37	1.28	(1.13, 1.46)	<0.001	1.19	(1.03, 1.38)	0.02

CKD, chronic kidney disease; ESRD, end-stage renal disease; PY: person-years; IR: incidence rate, per 1000 person-years; cHR, crude hazard ratio; aHR: adjusted hazard ratio; CI, confidence interval.

aHR[a]: multivariable analysis including sex, age, obesity, smoking status, comorbidities, CCI, DCSI scores, medications, number of oral antidiabetic drugs, and diabetes or hypertension duration.

Global Burden of Metabolic Risk Factors for Chronic Diseases Collaboration reported that high blood pressure accounts for 45-46% of CKD deaths (23). Our study showed that patients with diabetes and subsequent hypertension and patients with coexisting diabetes and hypertension with a previous history of hypertension exhibited a higher risk of incident CKD. Shear stress in hypertension may induce endothelial dysfunction, impair renal autoregulation, change renal blood flow, activate the renin-angiotensin-aldosterone system (RAAS), and result in CKD (20). A meta-analysis revealed that intensive blood pressure lowering strategies could significantly reduce the risk of albuminuria but with no significant lowering of ESRD risk (24). Patients with diabetes should avoid excessive dietary sodium, control obesity, engage in physical activity, and reduce alcohol consumption to mitigate hypertension development and attenuate CKD risk (25).

ESRD is a condition with GFR < 15 ml/min/1.73 m^2 or the need for dialysis or renal transplantation. Approximately 45% of patients with ESRD had type 2 diabetes in Taiwan (26). Up to 80% of ESRD was caused by diabetes, hypertension, or coexisting diabetes and hypertension (6). Both diabetes and hypertension are important prognostic factors for the progression of CKD to ESRD (20). A cohort study showed that the presence of diabetes could worsen patients with CKD to ESRD (27). The Multiple Risk Factor Intervention Trial established a consistent relationship between increased blood pressure and higher ESRD risk with the independence of relevant variables (28). Our study revealed that persons with diabetes and subsequent hypertension and patients with hypertension and subsequent diabetes showed a significantly higher risk of ESRD; especially persons with diabetes and subsequent hypertension had a very high adjusted HR [42.38(22.62-79.4)] for ESRD compared to persons without subsequent hypertension. Adding hypertension to persons with diabetes significantly increased the risk of ESRD. However, in patients with coexisting diabetes and hypertension, a previous history of diabetes seemed to have a higher impact on the risk of ESRD than a previous history of hypertension. This finding is consistent with previous reports that patients with a longer duration of diabetes showed a higher risk of ESRD (29). We must strive to mitigate the comorbidities of hypertension or diabetes to attenuate the progression of CKD to ESRD.

Approximately 35% of patients with type 2 diabetes have retinopathy. About 10% of patients with retinopathy have sight-threatening retinopathy (30) requiring close follow-up and aggressive treatments, such as vitrectomy, laser photocoagulation, or intravitreal anti-vascular endothelial growth factor injections to improve vision and avoid blindness. Taiwan Diabetes Atlas (2019) has reported that approximately 0.32% of persons with type 2 diabetes have sight-threatening retinopathy (31). The estimated global burden of retinopathy and sight-threatening retinopathy is 93 and 28 million individuals, respectively (30). Hypertension may worsen the progression of retinopathy (9), and suboptimal glycemic control may increase the retinopathy risk by 10–40% (29). Our study showed that persons with hypertension and subsequent diabetes and patients with comorbid diabetes and hypertension with a previous history of diabetes exhibited higher risks of sight-threatening retinopathy. Diabetes seems to play a crucial role in the development of sight-threatening retinopathy. However, patients with diabetes and subsequent hypertension also showed a significantly higher risk of sight-threatening retinopathy. Thus, the impact of hypertension on the risk of sight-threatening retinopathy cannot be ignored.

Inadequate treatment of foot ulcers or infection raises the risk of leg amputation, resulting in worsened quality of life in patients, reduced work performance, and impaired self-esteem (29). People with diabetes are 7–30 times more likely to receive non-traumatic leg amputations than the general population, accounting for more than half of all amputations (29). According to the Taiwan Diabetes Atlas (2019) report, approximately 1.16% of patients with type 2 diabetes had a diabetic foot, and 20.5% of these patients eventually needed leg amputations (31). Our study demonstrated that persons with diabetes and subsequent hypertension and persons with hypertension and subsequent diabetes showed an increased risk of leg amputation. We should strive to prevent subsequent hypertension development in patients with diabetes and subsequent diabetes development in patients with hypertension to reduce the risk of leg amputation in the future.

There are some disadvantages to this study. First, this dataset lacks information on blood pressure, glucose, hemoglobin A1C, renal function, urine protein, and retinal photographs to diagnose hypertension, diabetes, CKD, and retinopathy. We used the ICD codes to diagnose these diseases with acceptable accuracy, but there could have been potential errors. Some patients with mild or moderate retinopathy and mild renal dysfunction may escape detection with this protocol. Due to a lack of information on blood pressure and glucose, we attempted to match the numbers of antihypertensive drugs and antidiabetic drugs to balance the severity and treatment of hypertension and diabetes. Second, this administrative database lacks information on alcohol intake, family history, and physical activity. We tried to include more important variables, such as sex, age, obesity, smoking status, comorbidity, diabetes complication scores, and medications; we performed propensity score matching to increase the comparability between study and control groups. However, the unmeasured and unknown confounding factors still influenced our results. Third, the patients in this nationwide population-based study were mainly from Taiwan, and the results may not apply to other ethnicities. Finally, this study is a retrospective cohort study with some unobserved and unknown biases, and prospective studies are warranted to confirm our results.

In conclusion, CKD, retinopathy, and leg amputation are largely preventable and treatable diseases (19). Our study demonstrated that persons with diabetes and subsequent hypertension and persons with hypertension and subsequent diabetes showed significantly higher risks of incident CKD, ESRD, sight-threatening retinopathy, and leg amputation. This was rarely reported by previous studies (21). The family, school, and society should continuously educate people to avoid unhealthy lifestyles. Multifactorial interventions are necessary to mitigate comorbid hypertension or diabetes (28) and reduce the risk of nephropathy, retinopathy, and amputation.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Data of this study are available from the National Health Insurance Research Database (NHIRD) published by Taiwan National Health Insurance (NHI) Administration. The data utilized in this study cannot be made available in the paper. Requests for data can be sent as a formal proposal to the NHIRD Office (https://dep.mohw.gov.tw/DOS/cp-2516-3591-113.html) or by email to stsung@mohw.gov.tw.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of China Medical University and Hospital (CMUH109-109-REC2-031). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

F-SY, Y-HS, and C-MH participated in the study design. JC-CW, C-CH, and Y-HS participated in the study coordination and data collection. Y-HS and C-CH participated in the data analysis; all authors contributed to the interpretation of the results and the discussion. F-SY, JC-CW, and C-MH participated in manuscript writing; all authors participated in revising the manuscript. C-CH and C-MH are the guarantors of this work, and have full access to all the data in the study, and take responsibility for the integrity of the data and the accuracy of data analyses. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Lipidomics Reveals Serum Specific Lipid Alterations in Diabetic Nephropathy

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In diabetes mellitus (DM), disorders of glucose and lipid metabolism are significant causes of the onset and progression of diabetic nephropathy (DN). However, the exact roles of specific lipid molecules in the pathogenesis of DN remain unclear. This study recruited 577 participants, including healthy controls (HCs), type-2 DM (2-DM) patients, and DN patients, from the clinic. Serum samples were collected under fasting conditions. Liquid chromatography-mass spectrometry-based lipidomics methods were used to explore the lipid changes in the serum and identify potential lipid biomarkers for the diagnosis of DN. Lipidomics revealed that the combination of lysophosphatidylethanolamine (LPE) (16:0) and triacylglycerol (TAG) 54:2-FA18:1 was a biomarker panel for predicting DN. The receiver operating characteristic analysis showed that the panel had a sensitivity of 89.1% and 73.4% with a specificity of 88.1% and 76.7% for discriminating patients with DN from HCs and 2-DM patients. Then, we divided the DN patients in the validation cohort into microalbuminuria (diabetic nephropathy at an early stage, DNE) and macroalbuminuria (diabetic nephropathy at an advanced stage, DNA) groups and found that LPE(16:0), phosphatidylethanolamine (PE) (16:0/20:2), and TAG54:2-FA18:1 were tightly associated with the stages of DN. The sensitivity of the biomarker panel to distinguish between patients with DNE and 2-DM, DNA, and DNE patients was 65.6% and 85.9%, and the specificity was 76.7% and 75.0%, respectively. Our experiment showed that the combination of LPE(16:0), PE(16:0/20:2), and TAG54:2-FA18:1 exhibits excellent performance in the diagnosis of DN.

Keywords: Lipidomics, LPE(16:0), PE(16:0/20:2), TAG54:2-FA18:1, diabetic nephropathy

INTRODUCTION

As a significant microvascular complication of diabetes mellitus (DM), both type 1 and type 2, diabetic nephropathy (DN) has become the leading cause of chronic kidney disease (CKD) (1, 2). DN is characterized by dysfunction of the glomerular filtration barrier and decreased kidney function, which could be directly reflected by the persistent elevation of albumin in the urine and a progressive decrease in estimated glomerular filtration rate (eGFR), respectively (3). By 2019, there were approximately 463 million DM patients worldwide, among which type-2 DM (2-DM) accounted for more than 90% (4). It is estimated that 25–40% of diagnosed DM patients will eventually develop DN (5). Meanwhile, DN is an independent risk factor for increased mortality from cardiovascular causes, such as myocardial infarction, sudden cardiac death, stroke, and other fatal complications of diabetic cardiomyopathy (6).

In the clinic, microalbuminuria is considered the earliest evidence of the onset of DN. It has been reported that microalbuminuria progresses to macroalbuminuria in 50% of diagnosed DN patients without effective intervention and eventually develops into end-stage renal disease (ESRD) (7, 8). Undoubtedly, albuminuria is a significant sign of DN. However, the development of kidney impairment in DM patients is not synchronized with the increase in albuminuria (9). According to the national health and nutrition examination survey (NHANES), the number of DN patients with an eGFR of < 60 ml/min/1.73 m^2 but without albuminuria has increased over the past 30 years (10). In addition, these patients' annual mortality rate increased from 3.5% to 5.1% during this period (11). At present, the urine albumin creatine ratio (UACR) and eGFR are broadly applied parameters for diagnosing the initiation and progression of DN in the clinic. Nevertheless, in most DN patients during the early stages, their urinary albumin or eGFR level is normal. It has also been reported that the levels of microalbuminuria in some DN patients who received or did not receive intervention treatment returned to baseline rather than progressing to macroalbuminuria (12–14). Therefore, it is urgently necessary to develop more accurate diagnostic markers for DN in the clinical setting.

Lipid molecules are ubiquitous in all organisms and they make up essential components of cell membranes, lipid particles, and nerve myelin sheaths (15). Their functions include serving as cell barriers, membrane matrix, signal transduction, and energy storage (16). In 2005, the LIPID MAPS consortium classified lipids into eight categories based on their chemical and biochemical characteristics, which contains tens to hundreds of thousands of molecular species (17). Lipids are highly complex and dynamic, changing with physiological, pathological, and environmental conditions (18). In particular, lipid metabolites can serve as signaling molecules to activate multiple signaling pathways, thereby regulating cell growth, proliferation, and differentiation (19–21). Lipid disorders are associated with many diseases, such as Alzheimer's disease, metabolic disorders, cancer, and kidney disease (22–24). Lipidomics is the systematic analysis of lipids in the entire organism. It reveals the mechanism of lipids in various life activities (25). A previous urinary exosomal lipidomics study on DM and DN revealed that diacylglycerol (DAG), triacylglycerol (TAG), ganglioside GM3, and lysophosphatidylcholine (LPC) were significantly upregulated in DN patients (26).

In this study, we aimed to analyze the serum lipid characteristics in HCs, 2-DM patients, and DN patients by liquid chromatography-mass spectrometry metabolomics (LC–MS). The aim was to evaluate the effects of lipid metabolism on DN development, to understand the mechanisms of metabolic disorders in DN, and to identify potential lipid biomarkers for DN.

MATERIALS AND METHODS

Ethics Compliance Statement

All procedures were approved by the Institutional Review Board and the Ethics Committee of the First Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (2019NL-109–02), registered in the Chinese Clinical Trial Registry (ChiCTR2000028949), and followed the Declaration of Helsinki. After reviewing the study's written plan, all participants signed written informed consent before inclusion.

Study Population

A total of 577 participants, including healthy controls (HCs), patients with type 2 diabetes mellitus (2-DM), and diabetic nephropathy (DN), including microalbuminuria (diabetic nephropathy at an early stage, DNE) and macroalbuminuria (diabetic nephropathy at an advanced stage, DNA), from the Affiliated Hospital of Nanjing University of Chinese Medicine, were enrolled. All of the participants were Asian and met the diagnostic criteria of 2-DM, and the patients with DNE and DNA met the diagnostic criteria of DN. All serum samples were collected under fasting conditions, and the classification of DN was made according to UACR. In this study, we defined patients with UACR<30 mg/g as having 2-DM and 30≤UACR mg/g as having DN (30≤UACR ≤ 300 mg/g as having DNE, and UACR>300 mg/g as having DNA). The analytical sample included 169 healthy subjects, 170 participants with 2-DM, 238 participants with DN, including 64 participants with DNE, and 64 participants with DNA in the validation cohort. The clinical information of all participants, including all examination indicators, is recorded in **Table 1**. Serum samples were collected and stored at -80°C until further analysis.

Inclusion and Exclusion Criteria

Inclusion criteria include (1) 20-75 years old (2), All patients met the diagnostic criteria of 2-DM (3), The patients with microalbuminuria and macroalbuminuria met the diagnostic criteria of DN (4), eGFR >=90ml/min/1.73m2 in the 2-DM group, eGFR should be above 30ml/min/1.73m^2 in both microalbuminuria group and macroalbuminuria group (5), Blood pressure below 140/90 mmHg (6), sign the informed consent.

Exclusion criteria include (1) Primary kidney disease with a definite diagnosis (2), Other systemic diseases that can cause

TABLE 1 | Characterization of the study participants.

Covariate	Discovery Set (n = 330)			Validation Set (n = 247)			
	HCs	2-DM	DN	HCs	2-DM	DNE	DNA
Number	110	110	110	59	60	64	64
Male/Female	52/58	72/38	67/43	38/21	39/21	35/29	42/22
Age (years)	31.20 ± 8.4	53.75 ± 10.9	57.88 ± 10.2	34.47 ± 9.2	56.65 ± 10.9	53.38 ± 13.0	65.55 ± 12.2
BMI (kg/m^2)	21.71 ± 2.9	24.51 ± 5.3	25.61 ± 5.1	22.14 ± 2.9	25.18 ± 2.8	31.84 ± 44.9	25.94 ± 4.1
HbA1c (%)	—	6.2 ± 4.1	6.2 ± 4.0	—	8.8 ± 2.0	9.2 ± 2.0	7.6 ± 1.5
eGFR (ml/min/1.73m^2)	—	99.52 ± 14.0	74.75 ± 37.8	—	100.76 ± 14.0	99.89 ± 22.9	32.55 ± 25.7
ALB (g/L)	44.54 ± 2.4	38.88 ± 2.9	35.77 ± 6.0	42.03 ± 5.5	39.54 ± 4.3	38.90 ± 3.4	30.15 ± 4.7
BUN (mmol/L)	5 ± 1	7 ± 2	10 ± 6	5 ± 1	6 ± 2	7 ± 3	16 ± 7
Scr (μmol/L)	67 ± 12	68 ± 15	136 ± 152	68 ± 13	63 ± 12	67 ± 22	262 ± 172
Glu (mmol/L)	5 ± 0	8 ± 3	8 ± 3	5 ± 0	8 ± 3	10 ± 4	7 ± 4
Uric acid (μmol/L)	287 ± 69	308 ± 93	352 ± 141	289 ± 69	290 ± 97	326 ± 106	453 ± 121
Total cholesterol (mmol/L)	4 ± 1	4 ± 1	5 ± 2	5 ± 0	4 ± 1	5 ± 1	5 ± 2
Triglycerides (mmol/L)	1 ± 0	2 ± 4	2 ± 2	1 ± 0	2 ± 2	3 ± 3	2 ± 1
HDL cholesterol (mmol/L)	2 ± 0	1 ± 0	1 ± 0	2 ± 0	1 ± 0	1 ± 0	1 ± 0
LDL cholesterol (mmol/L)	2 ± 1	3 ± 1	3 ± 1	3 ± 0	3 ± 1	3 ± 1	3 ± 1
ACR (mg/g)	—	12.61 ± 5.8	1,160.07 ± 1,883.8	—	12.66 ± 7.7	69.81 ± 56.9	2,756.76 ± 2,087.4
24-hour urinary protein quantity (mg/24h)	—	37.92 ± 26.8	1,437.67 ± 2,298.2	—	48.36 ± 67.1	138.89 ± 295.2	3,555.62 ± 3,506.2

proteinuria (3), Acute complications of diabetes mellitus and urinary tract infection in the past 1 month (4), Complicated with serious primary diseases in cardiovascular, cerebrovascular, liver, kidney, and the hematopoietic system as well as the tumor (5), Suffering from mental illness and unable to cooperate (6), Pregnant or lactating women, or those preparing for pregnancy (7), Women in their menstrual period (8), Those who have participated in other clinical trials within the past 1 month.

Sample Preparation and Analysis

Serum samples were first thawed on ice. Briefly, 40 μL of serum was mixed with 225 μL of ice-cold MeOH. Each sample was then vortexed for 10 seconds and added to 750 μL of cold MTBE, and the mixtures were vortexed for 10 seconds before being shaken for 10 min at 4°C in an orbital mixer. After adding 188 μL of room-temperature LC/MS grade water, the samples were vortexed for 20 seconds and then centrifuged at 14,000 rcf at 4°C for 2 min. The upper liquid was transferred to fresh tubes and then dried in a SpeedVac sample concentrator at 45°C for 2 h. The dried lipids were redissolved in 100 μL of isopropyl alcohol/acetonitrile/water (30:65:5, *v/v/v*) mixture, and the samples were vortexed for 10 seconds and then centrifuged at 14,000 rcf at 4°C for 10 min. The mixture was then transferred to a sample vial with a glass insert and subjected to LC-MS analysis. Quality control (QC) samples were prepared by pooling equal amounts of lipid extracts from every sample, divided into aliquots, and analyzed every fifteen samples.

Chromatography and MS

The analysis was performed on a UHPLC system (Shimazu Nexera X2 LC-30AD, Japan) coupled with an ESI-triple quadrupole mass spectrometer (SCIEX Triple Quad 5500+, Singapore).

Lipid separation was carried out using a Waters ACQUITY UPLC BEH HILIC (100 mm×2.1 mm I.D., 1.7 μm; Waters, Milford, MA, USA) column at 35 ° C with a flow rate of 500 μL/min, and the injection volume of each sample was 5 μL.

The mobile phase consisted of two solvents: 10 mM ammonium acetate (NH_4OAc) in water: acetonitrile (5:95, *v/v*, pH adjustment usually not needed, A) and 10 mM ammonium acetate (NH_4OAc) in water: acetonitrile (50:50, *v/v*, adjusted pH 8.2 with ammonium hydroxide, B). The lipids were separated with an optimized gradient elution: 0–10.0 min, 0.1%–20% B; 10.0–11.0 min, 20%–98% B; 11.0–13.0 min, 98% B; 13.0–13.1 min, 98%–0.1% B; 13.1–16.0 min, 0.1% B.

The mass spectrometer was operated under positive and negative switching ionization mode with an electrospray voltage (capillary voltage) of 4500/-4500 V. The MRM/ retention time pairs were provided to the Scheduled MRM™ Algorithm to build the final MRM acquisition methods, and each MRM transition was monitored only during a short retention time window of 180 s. The typical source conditions were cohort: curtain gas as 35 and ion source temperature as 500 ° C. Ion source gas 1 (GS 1) and ion source gas 2 (GS 2) were all set at 50 and 60. The declustering potential was cohort at 80/-80 V. The collision cell exit potential was cohort at 9/-11 V in the positive or negative modes.

Data Analysis

Raw data were acquired from Analyst® 1.7.1 software (SCIEX) and then quantified with MultiQuant™ software. After removing the missing values using the 80% rule, the other missing values were replaced by 1/5 of each variable's minimum positive value. Furthermore, all statistical analyses were carried out on log-transformed data, which were median normalized and Pareto scaled before the multivariate analysis. All steps were completed by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). The identified lipids were further analyzed using univariate and multivariate statistical methods. The normalized data were imported into SIMCA software (version 14.1; Umetrics) and MetaboAnalyst 5.0 for partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), respectively. The significantly different

lipid metabolites were identified based on variable importance in the projection (VIP) obtained from the OPLS-DA model and Student's t-test (*p value*) with Benjamini-Hochberg-based false discovery rate (FDR). When the lipids met the criteria of VIP > 1.0, *p value* < 0.05 and FDR < 0.05 were considered differential metabolites.

Candidate metabolites were analyzed to identify potential diagnostic biomarkers. The forward stepwise binary logistic regression method and the Wald test were used to build the model based on the potential biomarkers. The diagnostic efficacy of the regression analysis results was analyzed and quantified by receiver operating characteristic (ROC) curve analysis. The area under the ROC curve (AUC) was calculated. Stepwise binary logistic regression and ROC curve analysis were performed with SPSS 25.0 software (SPSS, Inc.). GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) was used to visualize individual metabolite levels in violin graphs.

RESULTS

In this study, a total of 330 serum samples were collected as a discovery cohort to find candidate biomarkers. Meanwhile, a total of 247 participants, including 59 HCs, 60 patients with 2-DM, and 128 patients with DN, including 64 patients with DNE and 64 patients with DNA, were enrolled as a validation cohort to test the identified biomarkers (**Figure 1**). The demographic characteristics and clinical information of the subjects are shown in **Table 1**.

Serum Lipid Profiling of LC–MS

In the initial pseudotargeted lipid metabolomics analysis, we examined 330 serum samples. In the metabolic spectrum, 1221 metabolites were identified, covering more than 21 subclasses. We further applied PLS-DA (**Figure 2A**) and OPLS-DA (**Supplementary Figure S1**) to identify the metabolic profile differences between groups in the discovery data cohort. All of the QC samples clustered closely, verifying the reliability of the present study. Without overfitting of the model (**Supplementary Figure S2**), the apparent separation among the HCs, 2-DM, and DN groups, cumulative R2Y at 0.641 and Q2 at 0.359, indicated that the lipid metabolism pattern was changed among the three groups. Based on the significant changes in the comparison among the lipid metabolites of HCs, 2-DM, and DN, multivariate and univariate statistical significance criteria (VIP >1, *p value* < 0.05, and FDR< 0.05) were applied to determine 231 metabolites of 2-DM *vs.* HCs, 277 metabolites of DN *vs.* HCs, and 97 metabolites of DN *vs.* 2-DM. Among them, there were 15 differential metabolites in the three comparisons (**Figure 2B**).

Defining and Verifying Potential Biomarkers for DN

We then further examined the above metabolites in the validation cohort to identify potential biomarkers and test their validity. There were 47 metabolites (**Supplementary Table S1**) with significant differences in the three comparisons (2-DM *vs.* HCs, DN *vs.* HCs, and DN *vs.* 2-DM). Eight of these metabolites showed expression trends consistent with our findings in the discovery cohort, including LPE(16:0), LPE(18:0), LPE(20:1), PE (16:0/18:1), PE(16:0/18:2), PE(16:0/20:2), TAG54:2-FA18:1, and TAG54:3-FA18:0. Details of these metabolites are listed in **Table 2**. Subsequently, using the eight potential biomarkers, binary logistic regression analysis with a forwarding stepwise optimization algorithm (Wald) was used to construct the optimal model. Finally, the combination of LPE(16:0) and TAG54:2-FA18:1 was selected as the ideal biomarker panel to distinguish HCs, 2-DM, and DN. The ideal biomarker panel showed sensitivity at 61.7% and 89.1%, specificity at 86.4% and 88.1%, and AUC at 0.790 and 0.939, respectively, to differentiate patients with 2-DM and DN from HCs (**Figures 3A, B**). The ideal biomarker panel showed a sensitivity of 73.4%, specificity of 76.7%, and AUC of 0.808 to differentiate 2-DM and DN (**Figure 3C**). The predictive value was 75.0% for 2-DM *vs.* HCs

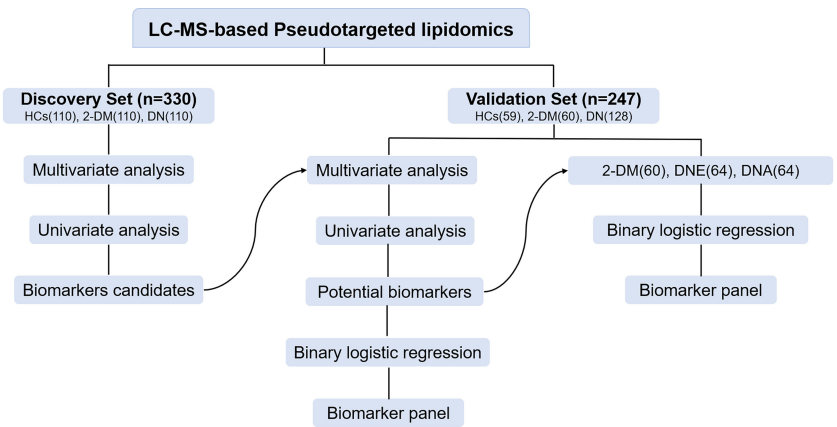

FIGURE 1 | Design of the study.

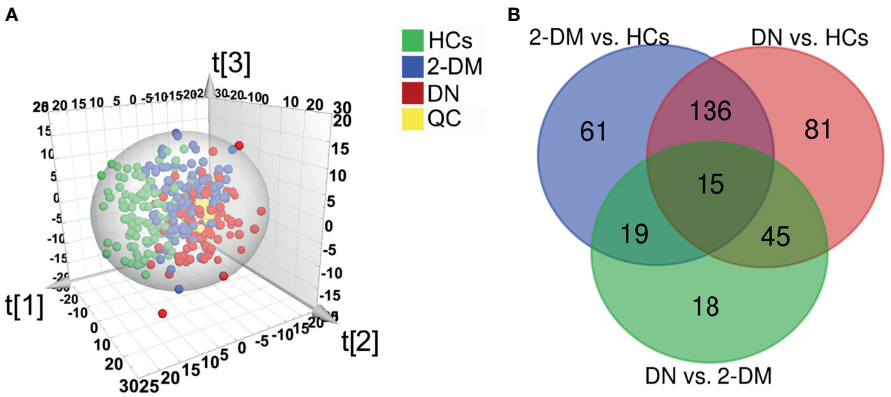

FIGURE 2 | Identification of potential metabolic biomarkers for the diagnosis of DN. **(A)** Partial least squares-discriminant analysis (PLS-DA) score plot based on HCs (green), 2-DM (blue), DN (red) groups, and QC samples (yellow) in the Discovery Set. **(B)** Venn diagram displays the differential metabolites when the 2-DM and DN groups were compared with the HCs, and the DN groups was compared with the 2-DM in the Discovery Set.

in the validation cohort (**Figure 3D**), 81.2% for DN *vs.* HCs in the validation cohort (**Figure 3E**), and 90.6% for DN *vs.* 2-DM in the validation cohort (**Figure 3F**).

Biomarkers for the Differential Diagnosis of DNE and DNA

We further divided participants with DN in the validation cohort into DNE and DNA to determine if there were ideal biomarkers among these potential biomarkers that could distinguish 2-DM, DNE, and DNA. First, a heat map was used to find the relative intensity distribution of the eight potential biomarkers in HCs, 2-DM, DNE, and DNA, as shown in **Figure 4**. The serum levels of these metabolites in HCs, 2-DM, DNE, and DNA increased with the severity of the disease. On this basis, eight potential biomarkers were used to perform binary logistic regression analysis using a forward stepwise optimization algorithm (Wald) for the construction of optimal models for DNE *vs.* 2-DM, DNA *vs.* 2-DM, and DNA *vs.* DNE. The results showed that the combination of LPE(16:0), PE(16:0/20:2), and TAG54:2-FA18:1 could distinguish 2-DM, DNE, and DNA very well. The ideal biomarker panel showed a sensitivity of 65.6%, specificity of 76.7%, and AUC of 0.765 to differentiate 2-DM and DNE (**Figure 5A**). Similarly, between 2-DM and DNA, we showed a sensitivity of 87.5%, specificity of 80.0%, and AUC of 0.909 (**Figure 5B**); between DNE and DNA, the sensitivity index was 85.9%, the specificity index was 75.0%, and the AUC index was 0.848 (**Figure 5C**). Predictive values of 82.8%, 70.3%, and 64.1% were found for DNE *vs.* 2-DM, DNA *vs.* 2-DM, and DNA *vs.* DNE in the validation cohort by setting 0.423, 0.675, and 0.609 as the optimal cutoff values (**Figures 5D–F**). LPE(16:0), PE (16:0/20:2), and TAG54:2-FA18:1 levels were gradually increased in the candidates from HCs, 2-DM, DNE, and DNA (**Figure 6**). To further validate candidates that might be useful in detecting DN, we analyzed the relationship between each lipid species and eGFR, Scr, and UAE. The analysis showed that LPE(16:0) and PE (16:0/20:2) were negatively correlated with eGFR (r=-0.2161, P<0.001; r=-0.5206, P<0.001). LPE(16:0) and PE(16:0/20:2) were positively correlated with Scr (r=0.1613, P=0.013; r=0.3816, P<0.001). PE(16:0/20:2) was positively correlated with UAE (r=0.3028, P<0.001). In addition, the association analysis between UAE, Scr or eGFR, and lipidomes showed no significant correlation.

TABLE 2 | Identified differential metabolites between the 2-DM, DNE, DNA and health controls.

Metabolite	2-DM *vs.* HCs				DN *vs.* HCs				DN *vs.* 2-DM			
	VIP	*p value*	FDR	FC	VIP	*p value*	FDR	FC	VIP	*p value*	FDR	FC
LPE(16:0)	1.397	0.003	0.011	1.580	2.364	<0.001	<0.001	6.825	2.665	<0.001	<0.001	4.320
LPE(18:0)	1.361	0.007	0.022	2.006	2.231	<0.001	<0.001	5.072	2.025	<0.001	<0.001	2.528
LPE(20:1)	1.511	<0.001	0.002	2.927	2.126	<0.001	<0.001	5.707	1.859	<0.001	0.003	1.950
PE(16:0/18:1)	2.315	<0.001	<0.001	9.994	2.683	<0.001	<0.001	28.153	2.830	<0.001	<0.001	2.817
PE(16:0/18:2)	2.146	<0.001	<0.001	7.734	2.506	<0.001	<0.001	18.622	2.645	<0.001	0.001	2.408
PE(16:0/20:2)	2.347	<0.001	<0.001	9.186	2.693	<0.001	<0.001	28.255	2.412	<0.001	<0.001	3.076
TAG54:2-FA18:1	1.903	<0.001	<0.001	3.437	2.267	<0.001	<0.001	7.493	1.450	<0.001	0.002	2.180
TAG54:3-FA18:0	1.821	<0.001	<0.001	2.666	2.327	<0.001	<0.001	4.425	1.102	0.001	0.019	1.660

VIP, variable importance in the projection; FC, fold change; FDR, false discovery rate.

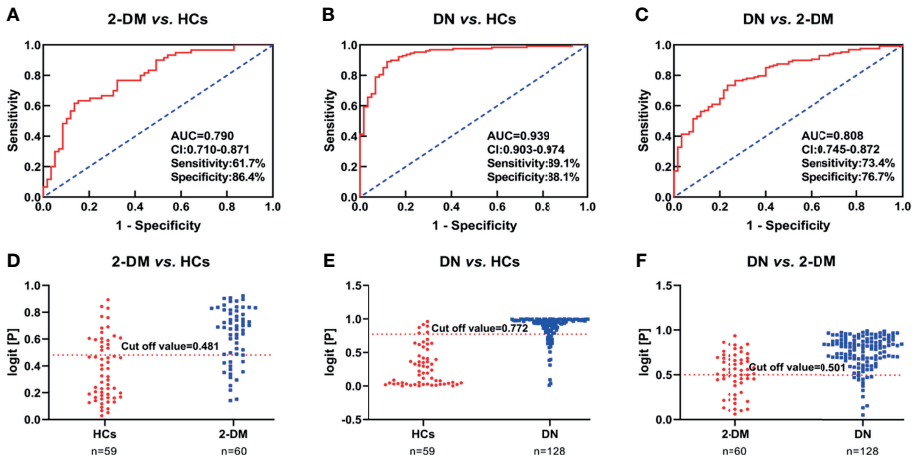

FIGURE 3 | **(A–C)** Receiver operating characteristic curve analysis (ROC) in combination with LPE(16:0) and TAG54:2-FA18:1 to discriminate HCs, 2-DM and DN patients in the Validation Set. **(D–F)** Prediction accuracies of the panel of biomarkers (LPE(16:0) and TAG54:2-FA18:1) in the Validation Set. The area under the curve (AUC) is given at 95 % confidence intervals. AUC, area under the curve; CI, confidence interval.

DISCUSSION

DN is a diabetic complication characterized by progressive kidney damage. Clinical treatment requires multimedication, and kidney replacement therapy imposes enormous economic burdens on the health care system (27). In this field, it is well known that DN patients have a higher mortality rate than DM patients without kidney damage (28). Therefore, early diagnosis and intervention to slow down the progression of DN will be of great significance to reduce the occurrence of unpredictable vascular events and to improve the survival rate and quality of life. DN is usually diagnosed as increased UACR and/or decreased eGFR, excluding primary and secondary CKD. Renal biopsy is the most accurate method for diagnosing DN, but in clinical practice, renal biopsy in DM patients is still rare because of its invasiveness (29). Since the accuracy and specificity of the current diagnostic criteria for DN cannot meet our requirements, an ideal diagnostic marker for DN, especially for the early stage of DN, is urgently needed. In this study, we performed a comprehensive study of lipids in the serum of HCs and 2-DM, DNE, and DNA individuals using pseudotargeted lipid metabolomics. A total of 1221 serum lipid metabolites were identified.

We then tested the lipid metabolites related to the occurrence and development of DN in the validation cohort. Compared with HCs and 2-DM patients, significantly increased levels of LPE (16:0), LPE(18:0), LPE(20:1), PE(16:0/18:1), PE(16:0/18:2), PE (16:0/20:2), TAG54:2-FA18:1, and TAG54:3-FA18:0 were observed in DN patients. Patients with CKD have previously been reported to exhibit disorders of glycerolipid metabolism and glycerophospholipid metabolism (30, 31).

PE(16:0/20:2) is a phosphatidylethanolamine(PE), which combinations of one chain of palmitic acid and one chain of eicosadienoic acid attached at the C-1 and C-2 positions, respectively. PE is the second most abundant and multifunctional glycerophospholipid in eukaryotic cells (32). It is essential in mammalian development and cellular processes, including being

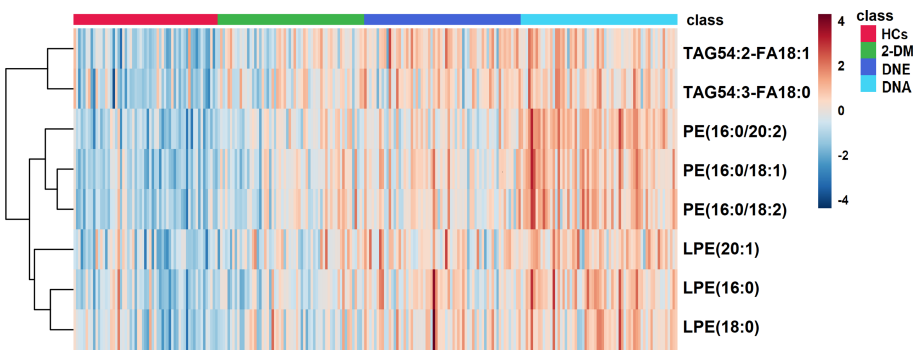

FIGURE 4 | A Heatmap of the differential metabolites in HCs, 2-DM, DNE and DNA. Rows: serum samples; Columns: lipid species.

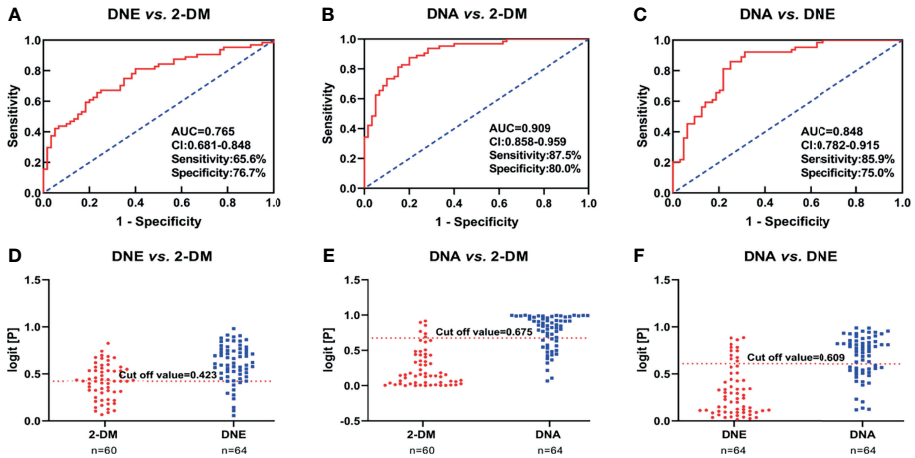

FIGURE 5 | **(A–C)** Receiver operating characteristic curve analysis (ROC) in combination with LPE(16:0) and TAG54:2-FA18:1 to discriminate HCs, 2-DM and DN patients in the Validation Set. **(D–F)** Prediction accuracies of the panel of biomarkers (LPE(16:0) and TAG54:2-FA18:1) in the Validation Set. The area under the curve (AUC) is given at 95 % confidence intervals. AUC, area under the curve; CI, confidence interval.

involved in metabolism and signaling (33). PE and cholesterol can improve the hardness of the bilayer membrane, which indicates that PE and cholesterol could maintain the fluidity of the cell membrane. Phosphatidylethanolamine n-methyltransferase (PEMT) is a crucial enzyme that promotes PC synthesis and PE conversion to PC. Once the PC: PE ratio is decreased, ER stress and SREBP1 are activated. ER stress is associated with insulin resistance (IR) and 2-DM (34, 35). Furthermore, once PE undergoes glycosylation due to the presence of free amine groups, it may increase the oxidation sensitivity in the case of hyperglycemic conditions (36). Additionally, to promote lipid peroxidation, glycated PE partially produces ROS, which is associated with inflammation and other DM complications, such as DN (37, 38).

When the PE: PC (phosphatidylcholine) ratio increases, the fluidity of the cell membrane decreases significantly. As a consequence, the increase in permeability of the cell membrane causes cell damage (39). This imbalance of the membrane lipid composition affects the characteristics of the membrane and induces pathological changes in erythrocyte membranes in patients with 2-DM (40).

Lysophosphatidylethanolamine (LPE) is a lysophospholipid product of partial hydrolysis of PE catalyzed by phospholipase A2 (PLA2) in glycerophospholipid metabolism (41). LPE(16:0) as an LPE, is mainly involved in the Phospholipid Biosynthesis. Investigation of existing literature, alteration of LPE (16:0) also was found in iron deficiency, ulcerative colitis, and colorectal cancer, but the specific mechanism of action remains unclear (42, 43). Before this, no such differences in the metabolism of LPE (16:0) have been reported in DM and DN. We speculated that LPE (16:0) might play a role in renal damage through its metabolites, basis the following information. LPE is converted to lysophosphatidic acid (LPA) by the action of lysophospholipase D (Lyso PLD). LPA can activate endothelial cells and initiate the secretion of a variety of proinflammatory peptides and proteins, in addition to causing the rupture of red blood cells and other cells, leading to hemolysis, cell necrosis, and organ damage, such as kidney disease (44). It has been reported in the literature that the LPA-LPAR axis mainly induces pathological changes in the structure and function of renal cells (45).

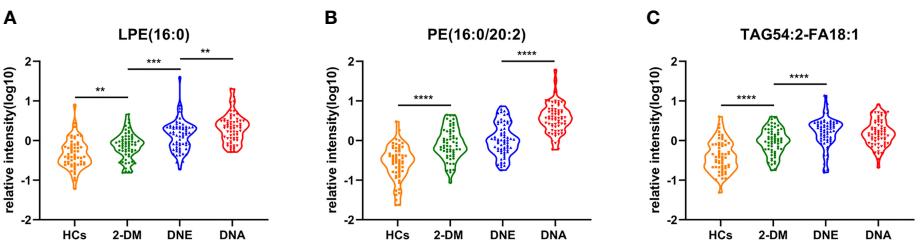

FIGURE 6 | Serum relative intensity of LPE(16:0) **(A)**, PE(16:0/20:2) **(B)**, and TAG54:2-FA18:1 **(C)** in the HCs (orange), 2-DM (green), DNE (blue) and DNA (red). **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Consistent with previous studies, the TAG level was elevated in patients with 2-DM and CKD compared to healthy subjects (46, 47). TAG biosynthesis occurs *via* the glycerolipid metabolic pathway of fatty acids (FAs) to produce LPA, which is further transformed into phosphatidic acid (PA). PA is then hydrolyzed to form diacylglycerols (DAGs) and finally esterified to TAGs (48, 49). It has been reported that TAG and DAG may contribute to insulin resistance by a similar mechanism as the stimulation of β-cell apoptosis by free fatty acids (FFAs) *via* c-Jun N-terminal kinase (JNK) (50). KEGG reactions in human pathways involving TAG54:2-FA18:1, Phospholipid + 1,2-Diacyl-sn-glycerol <=> Lysophospholipid + Triacylglycerol, verify the interconnection between PE, LPE, and TAG, and whether these metabolic changes broke the balance of this reaction, and then triggered a series of metabolic diseases. Unfortunately, the specific mechanism of which needs further research.

This lipid metabolomics provides a strategy for DN diagnosis in the clinic. The results can be used as a reference for further clinical examination. However, this study does have its limitations. First, all participants were Asian and enrolled from the same center, and because both 2-DM and DN were accompanied by obesity, resulting in significant differences between groups in terms of IBM and age, which may limit the applicability of our conclusions. Second, lipidomics analysis has limitations, and the results need to be further verified in additional studies. In future studies, the patients should be expanded to include other races and ethnicities across multiple research centers. The number of participants should be increased and information on their renal function parameters should be followed up to make the results more compelling.

In summary, we found that lipid metabolism disorders in DN were associated with LPE, PE, and TAG changes. A biomarker panel comprised of LPE(16:0), PE(16:0/20:2), and TAG54:2-FA18:1 was identified and further validated by a longitudinal sectional study for the diagnosis of DN, which showed that LPE (16:0), PE(16:0/20:2), and TAG54:2-FA18:1 were positively correlated with the severity of the development of DN. This biomarker panel can identify DN patients and distinguish DNA and DNE patients from HCs and 2-DM individuals. Therefore, it is proposed that this lipid biomarker panel has great potential in the diagnosis and treatment of DN in the clinical setting.

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 Institutional Review Board and the Ethics Committee of the First Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TX, Conceptualization, Formal analysis, and Writing - Original Draft Preparation. XX and LuZ, Methodology, Data curation, and Writing - Review & Editing. KZ, QW, YY, and LiZ, Formal analysis and Validation. LL, LX, WQ, JW, and MK Investigation and Resources. XA, Funding acquisition; SL, Conceptualization, Project administration, and Funding acquisition. 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.2021.781417/full#supplementary-material

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frontiers in Endocrinology

ORIGINAL RESEARCH
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Development and Validation of a Prediction Model for Survival in Diabetic Patients With Acute Kidney Injury

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Objective: We aimed to analyze the risk factors affecting all-cause mortality in diabetic patients with acute kidney injury (AKI) and to develop and validate a nomogram for predicting the 90-day survival rate of patients.

Methods: Clinical data of diabetic patients with AKI who were diagnosed at The First Affiliated Hospital of Guangxi Medical University from April 30, 2011, to April 30, 2021, were collected. A total of 1,042 patients were randomly divided into a development cohort and a validation cohort at a ratio of 7:3. The primary study endpoint was all-cause death within 90 days of AKI diagnosis. Clinical parameters and demographic characteristics were analyzed using Cox regression to develop a prediction model for survival in diabetic patients with AKI, and a nomogram was then constructed. The concordance index (C-index), receiver operating characteristic curve, and calibration plot were used to evaluate the prediction model.

Results: The development cohort enrolled 730 patients with a median follow-up time of 87 (40–98) days, and 86 patients (11.8%) died during follow-up. The 90-day survival rate was 88.2% (644/730), and the recovery rate for renal function in survivors was 32.9% (212/644). Multivariate analysis showed that advanced age (HR = 1.064, 95% CI = 1.043–1.085), lower pulse pressure (HR = 0.964, 95% CI = 0.951–0.977), stage 3 AKI (HR = 4.803, 95% CI = 1.678–13.750), lower 25-hydroxyvitamin D3 (HR = 0.944, 95% CI = 0.930–0.960), and multiple organ dysfunction syndrome (HR = 2.056, 95% CI = 1.287–3.286) were independent risk factors affecting the all-cause death of diabetic patients with AKI (all $p < 0.01$). The C-indices of the prediction cohort and the validation cohort were 0.880 (95% CI = 0.839–0.921) and 0.798 (95% CI = 0.720–0.876), respectively. The calibration plot of the model showed excellent consistency between the prediction probability and the actual probability.

Conclusion: We developed a new prediction model that has been internally verified to have good discrimination, calibration, and clinical value for predicting the 90-day survival rate of diabetic patients with AKI.

Keywords: diabetes, acute kidney injury, prognosis, nomogram, prediction model

INTRODUCTION

In recent years, the incidence of diabetes has increased globally. According to the International Diabetes Federation Atlas, 9th edition, 463 million adults worldwide live with diabetes as of 2019, with a prevalence rate of approximately 9.3% and an average annual growth rate of 51% (1). Diabetes easily leads to several complications that affect the prognosis of patients with diabetes (2, 3). Approximately 4.2 million people worldwide died from diabetes or its complications in 2019, accounting for approximately 11.3% of all-cause deaths worldwide (1).

Patients with diabetes often develop acute kidney injury (AKI) due to poor blood glucose control, infection, organ failure, contrast agents, and reduced resistance (4, 5). A large retrospective cohort study has shown that the incidence of AKI is 48.6% in diabetic patients, which is significantly higher than that in non-diabetic patients (17.2%) (6). Diabetes can increase the incidence of AKI and the risk of poor renal outcomes (7). Diabetic patients with AKI without timely treatment will progress to chronic renal failure (CRF) and even end-stage renal disease (ESRD), which should be treated by renal replacement therapy (RRT). One study has shown that the RRT rate of AKI in diabetic patients is approximately 5-fold higher than that in non-diabetic patients (8). AKI is not only a common complication of diabetes but also an independent risk factor associated with the survival rate and CRF of diabetic patients (9–11). Diabetic patients with AKI have poorer clinical outcomes (12). Therefore, early identification and intervention of risk factors affecting clinical outcomes can help to delay the progression and improve the survival rate of diabetic patients with AKI.

However, there have been few studies on the factors affecting the prognosis of diabetic patients with AKI. Due to the high prevalence and poor prognosis of AKI in diabetic patients, it is necessary to develop a prognostic model for diabetic patients with AKI. A nomogram is considered a reliable tool that can be used to create a simple intuitive predictive model that quantifies the risk of a clinical event (13, 14). In the present study, an accurate and beneficial prediction model based on a nomogram for predicting the 90-day survival rate of diabetic patients with AKI was developed, aiming to explore the risk factors for poor short-term prognosis and to provide a reference for the prevention and treatment of diabetic patients with AKI.

MATERIALS AND METHODS

Subjects

All subjects were patients treated at The First Affiliated Hospital of Guangxi Medical University from April 30, 2011 to April 30, 2021, who were diagnosed with diabetes and AKI. The inclusion criteria were as follows: 1) a clear diagnosis of diabetes before AKI and 2) changes in serum creatinine (Scr) consistent with the diagnostic criteria for AKI. The exclusion criteria were as follows: 1) age <18 years; 2) patients diagnosed with stage 5 chronic kidney disease (CKD) or who received regular RRT; 3) incomplete baseline data; and 4) patients lost to follow-up within 90 days of AKI diagnosis. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Guangxi Medical University [approval no. 2019 (KY-E-028)]. As this was a retrospective analysis of anonymized clinically obtained data and all patient identifiers were removed, there was no need for patients to sign an informed consent form. The present study was conducted in accordance with the tenets of the Declaration of Helsinki (15).

Research Methods and Groupings

We followed the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) statement for reporting multivariable prediction model development and validation (16, 17). The TRIPOD checklist of the present study is found in the **Supplementary Material**. A retrospective cohort study was performed, and diabetic patients with AKI were followed up for 90 days or death (death within 90 days). The primary study endpoint was all-cause death within 90 days of AKI diagnosis. Patients were randomly divided into a development cohort and a validation cohort at a ratio of 7:3. The development cohort was used to construct the prediction model, and the validation cohort was used to verify the prediction accuracy of the model.

Data Collection

Clinical parameters and demographic data were collected, including age, sex, diabetes duration, complications, smoking, drinking, body mass index (BMI), blood pressure, baseline levels of routine blood tests, liver function, renal function, electrolytes, myocardial enzymes, N-terminal prohormone of brain natriuretic peptide (NT-proBNP), 25-hydroxyvitamin D3 [25(OH)D3], AKI stage, infection, heart failure, cerebrocardiovascular diseases, and multiple organ dysfunction syndrome (MODS). The baseline Scr was defined as a stable Scr within the last 3 months or longer if none was available within 3 months (18). ΔScr was calculated as the Scr difference at the end of follow-up and baseline.

Diagnostic Criteria

The diagnosis of diabetes in our institution complies with the World Health Organization criteria as follows: diabetic symptoms and 1) random blood glucose ≥11.1 mmol/L, 2) fasting blood glucose (FBG) ≥7.0 mmol/L, or 3) postprandial

blood glucose (PBG) ≥11.1 mmol/L (19). AKI was diagnosed in accordance with the diagnostic criteria in the guidelines of KDIGO as follows: increase in Scr ≥26.5 μmol/L within 48 h or an increase from the baseline value by ≥50% within 7 days (20). The criteria of AKI stages were as follows: stage 1, AKI was defined by the AKI Network as at least a ≥50% rise or a ≥0.3 mg/dl rise from baseline Scr; stage 2, AKI was defined as a doubling in Scr from baseline; and stage 3, AKI was defined as a tripling in Scr from baseline or receiving acute dialysis during the hospital stay (21). Heart failure complied with the European Society of Cardiology guidelines as follows: the symptoms and/or signs of heart failure with left ventricular ejection fraction less than 40% (22). MODS was defined as acute and potentially reversible dysfunction of two or more organ systems (23).

Statistical Analysis

Statistical analyses were performed and graphics were produced with SPSS version 22.0 (IBM Corp., Armonk, NY, USA) and R software version 4.0.3 (https://www.r-project.org/). For continuous variables, data are presented as the mean ± standard deviation (normal distribution) or median and interquartile range (abnormal distribution). For dichotomous variables, data are presented as whole numbers and proportions [*n*(%)]. *T*-tests, chi-square tests, or Wilcoxon rank-sum tests were used to compare differences in the clinical data between the development cohort and the validation cohort by SPSS. Univariate Cox regression was used to screen the risk factors affecting the prognosis, and the "forward LR" method was then used to screen the variables of $p < 0.05$ that were included in the multivariate Cox proportional hazards regression model. Based on the results of multivariate Cox analysis, the "rms" package in R statistical software was used to construct the nomogram according to the hazard ratio (HR) and 95% confidence interval (95% CI) of the risk factors. Bootstrapping resampling techniques with 1,000 replications were used to perform internal validation. The concordance index (C-index) and receiver operating characteristic (ROC) curve were used to evaluate the differentiation of the prediction model in the development and validation cohorts. A C-index or area under the ROC curve (AUROC) >0.70 indicated that the prediction effect of the model was good. Calibration plots were drawn to evaluate the accuracy of the prediction model in the development and validation cohorts. The "rms" package was used to draw the calibration plots. Regarding the model, calibration lines closer to the standard line indicate better calibration degree of the model. A $p < 0.05$ was considered statistically significant in all analyses.

RESULTS

Characteristics of Patients in the Development and Validation Cohorts

There were 1,254 patients diagnosed with diabetic AKI, of whom 52 were younger than 18 years, 98 received regular RRT, 36 had incomplete baseline data, and 26 were lost to follow-up. Finally, a total of 1,042 patients were enrolled in our study, with 730 and 312 patients assigned to the development and validation cohorts, respectively. In the development cohort, 21.2% (n = 155) had stage 1 AKI, 24.4% (n = 178) had stage 2 AKI, and 54.4% (n = 397) had stage 3 AKI; the median follow-up time was 87 (40–98) days. By the end of follow-up, 86 patients had died (11.8%) within 90 days of AKI diagnosis. The 90-day cumulative survival rate was 88.2% (644/730), and the recovery rate for renal function in survivors was 32.9% (212/644). The main causes of death were cerebrocardiovascular diseases in 37 cases (43.0%), bacterial infection in 28 cases (32.6%), and other or unknown causes in 21 cases (24.4%). In the validation cohort, the median follow-up time was 86.5 (36–99) days. Forty patients died (12.8%) within 90 days of AKI diagnosis. The 90-day cumulative survival rate was 87.2% (272/312), and the recovery rate for renal function in survivors was 33.8% (92/272). The main causes of death were cerebrocardiovascular diseases in 17 cases (42.5%), bacterial infection in 14 cases (35.0%), and other or unknown causes in 9 cases (22.5%).

Table 1 shows the patient characteristics by cohort. Compared to the validation cohort, patients in the development cohort had higher creatine kinase and lower endogenous creatinine clearance ($p < 0.05$). There was no significant difference in sex, age, diabetes duration, BMI, blood pressure, white blood cell count (WBC), platelets, hemoglobin, 25(OH)D3, creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), NT-proBNP, FBG, PBG, glycosylated hemoglobin A1c (HbA1c), blood urea nitrogen (BUN), baseline Scr, uric acid (UA), serum kalium levels, incidence of RRT, heart failure, CKD, bacterial infections, or MODS ($p > 0.05$). Kaplan–Meier survival analysis showed that there was no significant difference in the 90-day survival rates between the development and validation cohorts (log rank $\chi^2 = 0.208$, $p = 0.648$).

Risk Factors Affecting Prognosis

Cox regression analysis was used to construct the prediction model because the Cox proportional hazards assumption was met. As shown in **Table 2**, univariate Cox analysis of the development cohort revealed that advanced age, pulse pressure, WBC, NT-proBNP, ΔScr, AKI stage, serum kalium levels, 25(OH)D3, heart failure, and MODS were related factors for all-cause death of diabetic patients with AKI ($p < 0.05$). Sex, diabetes duration, length of hospital stay, smoking, drinking, BMI, blood pressure, platelets, hemoglobin, CK-MB, LDH, FBG, PBG, HbA1c, BUN, baseline Scr, UA, incidence of proteinuria, RRT, bacterial infection, CKD, coronary heart disease, and cerebrovascular disease were not correlated with death ($p > 0.05$). Hence, these significant indicators [advanced age, pulse pressure, WBC, NT-proBNP, ΔScr, AKI stage, serum kalium levels, 25(OH)D3, heart failure, and MODS] with statistical significance from the univariate analysis were included in the multivariate Cox regression analysis. The results showed that advanced age (every 1 year increase: HR = 1.064, 95% CI = 1.043–1.085, $p < 0.001$), stage 3 AKI (HR = 4.803, 95% CI = 1.678–13.750, $p = 0.003$), and MODS (HR = 2.056, 95% CI = 1.287–3.286, $p = 0.003$) were independent risk factors affecting the all-cause death of diabetic patients with AKI, while higher pulse pressure (every 1 mmHg increase: HR = 0.964, 95% CI = 0.951–0.977, $p < 0.001$) and higher 25(OH)D3 (every 1 nmol/L increase:

TABLE 1 | Differences in the development cohort and the validation cohort in terms of demographic characteristics and laboratory values.

Parameters	Development cohort	Validation cohort	$t/\chi^2/z$	*p*-value
Male/female	491/239	217/95	0.527	0.468
Age (years)	62.54 ± 13.92	62.32 ± 14.28	0.229	0.819
Diabetes duration (months)	73 (25–124)	75 (27–119)	0.108	0.912
BMI (kg/m^2)	24.02 ± 4.00	23.93 ± 4.31	0.258	0.796
SBP (mmHg)	133.75 ± 27.33	135.42 ± 25.44	–0.911	0.363
DBP (mmHg)	76.12 ± 16.50	76.23 ± 15.06	–0.101	0.920
PP (mmHg)	57.63 ± 18.67	59.19 ± 18.89	–1.215	0.224
WBC ($\times10^9$/L)	12.84 ± 7.70	12.78 ± 7.63	0.120	0.905
Hb (g/L)	102.39 ± 23.83	104.47 ± 24.38	–1.220	0.223
PLT ($\times10^9$/L)	187.65 ± 87.16	194.40 ± 87.19	–1.090	0.276
NEU	0.76 ± 0.16	0.75 ± 0.16	1.002	0.317
Alb (g/L)	31.76 ± 7.56	32.01 ± 7.70	–0.451	0.652
25(OH)D3 (nmol/L)	54.36 ± 23.38	55.96 ± 22.88	–1.018	0.309
CK (U/L)	211 (86–345)	124 (59–351)	–2.881	0.004
CK-MB (U/L)	29.31 ± 26.98	29.25 ± 25.88	0.030	0.976
LDH (U/L)	488.39 ± 496.04	496.29 ± 473.91	–0.223	0.823
NT-proBNP (pg/ml)	5,175.09 ± 3,073.04	5,151.26 ± 3,092.85	0.106	0.915
FBG (mmol/L)	8.42 ± 3.52	8.49 ± 3.46	–0.265	0.791
PBG (mmol/L)	12.44 ± 3.85	12.54 ± 4.12	–0.242	0.809
HbA1c (%)	8.03 ± 2.80	8.26 ± 2.94	–0.946	0.344
BUN (mmol/L)	14.60 ± 9.82	13.35 ± 10.09	1.850	0.065
baseline Scr (μmol/L)	143.08 ± 123.60	136.92 ± 112.34	0.629	0.530
UA (μmol/L)	418.72 ± 198.97	412.70 ± 211.12	0.430	0.667
HCO_3^- (mmol/L)	21.88 ± 5.47	22.05 ± 5.34	–0.440	0.660
Ccr (ml/min)	42.23 ± 25.46	46.38 ± 29.00	–1.991	0.047
Cys-C (mg/L)	2.40 ± 1.48	2.28 ± 1.46	1.163	0.245
ΔScr (μmol/L)	131.59 ± 190.11	115.78 ± 179.67	1.250	0.212
Serum kalium (mmol/L)	4.31 ± 1.75	4.28 ± 0.93	0.234	0.815
RRT, *n* (%)	163 (22.3)	64 (20.5)	0.286	0.593
Bacterial infection, *n* (%)	506 (69.3)	209 (67.0)	0.550	0.458
HF, *n* (%)	257 (35.2)	109 (34.9)	0.007	0.933
CKD, *n* (%)	189 (25.9)	78 (25.0)	0.091	0.763
MODS, *n* (%)	114 (15.6)	50 (16.0)	0.028	0.868
Death, *n* (%)	86 (11.8)	40 (12.8)	0.222	0.637

25(OH)D3, 25-hydroxyvitamin D3; Alb, albumin; BMI, body mass index; BUN, blood urea nitrogen; Ccr, endogenous creatinine clearance rate; CK, creatine kinase; CK-MB, creatine kinase-MB; Cys-C, serum cystatin C; DBP, diastolic blood pressure; FBG, fasting blood glucose; FIB, fibrinogen; Hb, hemoglobin; HbA1c, glycosylated hemoglobin A1c; HF, heart failure; LDH, lactate dehydrogenase; MODS, multiple organ dysfunction syndrome; NEU, neutrophil percentage; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; PBG, postprandial blood glucose; PLT, platelet count; PP, pulse pressure; RRT, renal replacement therapy; SBP, systolic blood pressure; Scr, serum creatinine; UA, uric acid; WBC, white blood cell count; ΔScr, creatinine difference at the end of follow-up therapy.

TABLE 2 | Analysis of risk factors for prognosis in the development cohort (univariate and multivariate Cox regression).

Variables	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age	1.049 (1.032–1.067)	<0.001	1.064 (1.043–1.085)	<0.001
Pulse pressure	0.984 (0.972–0.996)	0.009	0.964 (0.951–0.977)	<0.001
WBC	1.034 (1.010–1.058)	0.004		
ΔScr	1.002 (1.001–1.003)	<0.001		
AKI stage				
1	1 [Reference]	0.001	1 [Reference]	0.001
2	4.104 (1.687–9.983)	0.002	2.259 (0.769–6.632)	0.138
3	4.833 (2.075–11.259)	<0.001	4.803 (1.678–13.750)	0.003
Serum kalium	1.088 (1.043–1.135)	<0.001		
NT-proBNP	1.001 (1.000–1.001)	<0.001		
25(OH)D3	0.950 (0.937–0.962)	<0.001	0.944 (0.930–0.960)	<0.001
HF	2.272 (1.476–3.497)	<0.001		
MODS	4.178 (2.714–6.432)	<0.001	2.056 (1.287–3.286)	0.003

The forward method was used to screen variables.

25(OH)D3, 25-hydroxyvitamin D3; HF, heart failure; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; WBC, white blood cell count; ΔScr, serum creatinine difference at the end of follow-up and baseline.

HR = 0.944, 95% CI = 0.930–0.960, $p < 0.001$) were independent protective factors affecting the all-cause death of diabetic patients with AKI.

Development and Validation of the Prediction Model

A prediction model that incorporated the above independent predictors was developed as the nomogram (**Figure 1**). The 90-day survival rate after the diagnosis of AKI was estimated by calculating risk factor scores. Taking a 65-year-old patient with MODS as an example, the scores of each influencing factor were calculated as follows: 57.5 points for a 65-year-old, 37.5 points for a pulse pressure of 85 mmHg, 26.25 points for stage 3 AKI, 65 points for 25(OH)D3 of 55 nmol/L, and 13.75 points for MODS. The total score of this patient was 200 points (57.5 + 37.5 + 26.25 + 65 + 13.75), and the predicted 90-day survival rate was approximately 42%.

The C-index of the prediction model in the development cohort was 0.880 (95% CI = 0.839–0.921). As shown in **Figure 2A**, the AUROC of the prediction model for the 90-day survival rate was 0.860, and the sensitivity and specificity were 0.766 and 0.937, respectively. The C-index of the prediction model in the validation cohort was 0.798 (95% CI = 0.720–0.876) according to the internal verification by Bootstrap. As shown in **Figure 2B**, the AUROC of the prediction model for the 90-day survival rate was 0.774, and the sensitivity and specificity were 0.705 and 0.821, respectively. The risks of death in the decile groups were calibrated using a smoothing function, with the *X*-axis as the predicted probabilities and the *Y*-axis as the actual probabilities. In both the development and validation groups, the calibration plots of the prediction model were close to a straight line with a slope of 1 (**Figures 3A, B**).

DISCUSSION

The prognosis of diabetic patients with AKI is worse than that of non-diabetic or non-AKI patients (12, 24, 25). Therefore, it is particularly important to explore the risk factors affecting the clinical outcomes and to construct a prognostic model for diabetic patients with AKI. In the present study, the risk factors for short-term prognosis were evaluated using Cox regression analysis, and a prediction model of prognostic risk was constructed based on the clinical parameters and demographic characteristics of diabetic patients with AKI. The results of our study showed that the 90-day survival rate was 88.2%, and advanced age, lower pulse pressure, stage 3 AKI, lower 25(OH)D3, and MODS were independent risk factors affecting the all-cause death of diabetic patients with AKI. Based on these risk factors, a model was established to predict the short-term survival of diabetic patients with AKI. In addition, calibration plots, the C-index of the validation cohort, the AUROC of the validation cohort, and bootstrapping resampling techniques were used for the internal validation of the predictive model. The accuracy verification showed that the model had a certain predictive ability.

Several studies have shown that ketoacidosis, hyperosmolar and hyperglycemic coma, rhabdomyolysis, contrast agents, sepsis, and heart failure are risk factors for the development of AKI in diabetes mellitus (26, 27). If AKI is not corrected in time, the degree of kidney injury might be aggravated. AKI is associated with poor prognosis in patients, including the occurrence of CKD, progression of CKD, prolonged hospital stays, increased adverse cardiovascular events, and mortality (28–31). Previous studies on predictive models of AKI have mainly focused on specific populations of patients with AKI after cardiac surgery (32, 33), AKI after non-cardiac surgery (34, 35), septic AKI (36, 37), tumor-related AKI (38), and critical AKI (39). James et al. (40) constructed a predictive model of progression to advanced CKD after discharge in patients with AKI, and the results showed that older age, female sex, higher baseline Scr, higher baseline proteinuria, more severe AKI, and higher Scr at discharge were associated with a higher risk of progression to advanced CKD. However, few studies have developed or validated prediction models for all-cause mortality in diabetic patients with AKI. To our knowledge, this is the first study to develop a prognostic model for the 90-day survival rate in diabetes with AKI, which can help identify risk factors for poor prognosis in diabetes with AKI at an early stage and improve the short-term prognosis through timely interventions.

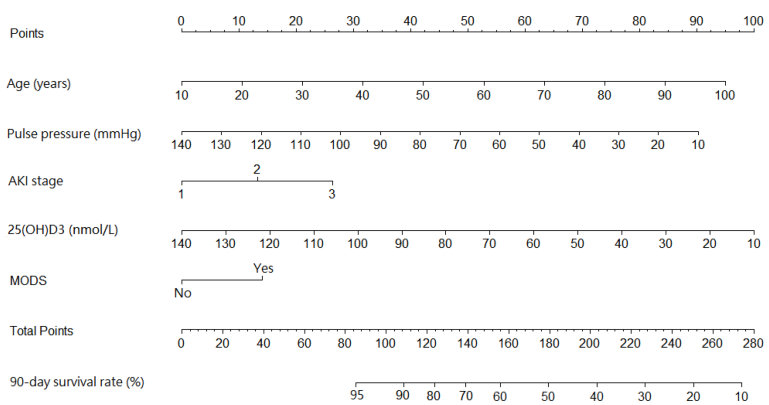

FIGURE 1 | Nomogram predicting the 90-day survival rate in diabetic patients with acute kidney injury (AKI).

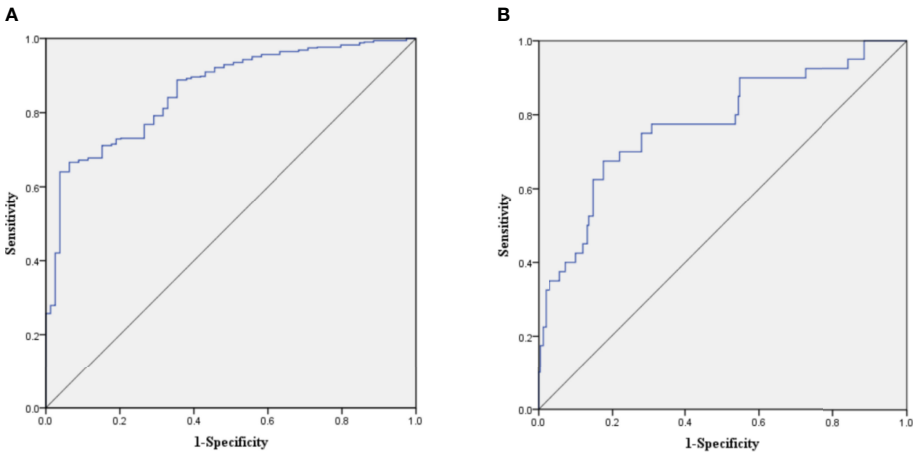

FIGURE 2 | **(A)** Receiver operating characteristic (ROC) curve of the prediction model in the development cohort. **(B)** ROC curve of the prediction model in the validation cohort.

The results of our study showed that 54.4% of patients had stage 3 AKI, indicating that this prediction model might be more suitable for predicting the prognosis of diabetic patients with AKI in more severe AKI stages. For patients with stage 1 or 2 AKI, its predictive effect still needs to be further explored. In addition, our study showed that more severe AKI was an important risk factor for increased all-cause mortality in diabetic AKI patients (HR = 4.803). Previous studies have shown that the overall in-hospital mortality rates are 0.6% in no AKI, 5.3% in stage 1 AKI, 13.4% in stage 2 AKI, and 35.4% in stage 3 AKI (41, 42), which also supported our study. Therefore, the short-term prognosis of diabetes with AKI can be preliminarily evaluated and predicted according to the stage of AKI in clinical practice.

Previous studies have shown that advanced age is an important risk factor for the occurrence and development of patients with diabetes mellitus and AKI (43, 44). Our study also showed that advanced age was an independent risk factor for all-cause mortality in diabetic patients with AKI. A previous animal study has shown that elderly type 2 diabetes mellitus (T2DM) rats have a greater decrease in medullary blood flow and glomerular filtration rate after renal ischemia reperfusion than middle-aged T2DM rats. The expression of renal adhesion molecules and the number of infiltrating immune cells in elderly T2DM rats are higher than those in young or middle-aged rats (45). A large multicenter cohort study of 72,310 elderly patients with T2DM has shown that congestive heart failure, cerebrovascular diseases, and mortality significantly increase with increasing age (46). With age, the physiological function, self-regulation, and reserve ability of the human body decrease. In addition, the decrease in arterial wall elasticity and compliance may also aggravate vascular damage, other complications, and

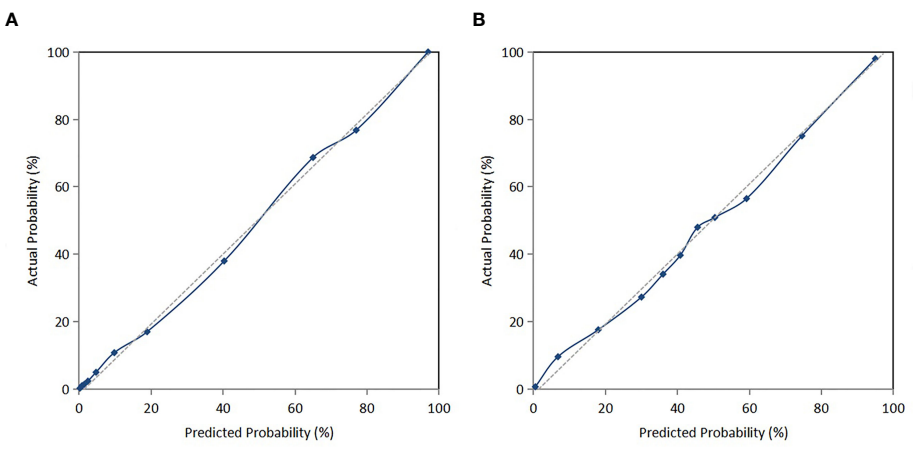

FIGURE 3 | **(A)** Calibration curve of the nomogram in the development cohort. **(B)** Calibration curve of the nomogram in the validation cohort.

the incidence of clinical events in advanced-age diabetic patients with AKI.

Our study showed that a lower 25(OH)D3 was a risk factor for all-cause mortality in diabetic patients with AKI. Fernandez-Juarez et al. (47) followed up 133 patients with T2DM with proteinuria, and they reported that a low 25(OH)D3 is associated with poor prognosis (Scr increase >50%, ESRD, and mortality). A previous study has also shown a strong association between vitamin D deficiency and the increased risk of heart failure in older patients (OR = 12.19), which was similar to the result of our study (48). An animal study has shown that activation of vitamin D receptors might alleviate cisplatin-induced AKI by inhibiting iron death (49). Vitamin D3 supplementation ameliorates kidney injury induced by hyperglycemia in diabetic mice by regulating lipid metabolism, oxidative stress, apoptosis, and autophagy (50). Therefore, a lower 25(OH)D3 might contribute to all-cause mortality in diabetic patients with AKI by increasing the risk of renal damage and cardiovascular events. In addition, our study also showed that MODS was another independent risk factor for all-cause mortality in diabetic patients with AKI. A retrospective study has shown that MODS is an independent risk factor for poor prognosis in hospital-acquired AKI (OR = 3.538), which was consistent with our study (51). Hemodynamic instability and volume overload are common in patients with MODS, and the mortality of MODS is approximately 40%, which increases with the number of failing organs (52). Thus, MODS might increase the risk of all-cause mortality in diabetic patients with AKI.

Limitations

The present study had several limitations. Firstly, the sample size was small and from only one medical center. We only verified the model internally in the same center, and the conclusions should be further confirmed by external validation at other centers. Secondly, the diagnosis of AKI in this study was only based on the criterion of Scr, which did not include the diagnostic criterion of urine volume. Thus, some patients may have been missed. Thirdly, the present study may have missed a few potential risk factors, such as microalbuminuria, albumin-to-creatinine ratio, drugs, and other therapeutic measures. Therefore, this prediction model still requires collecting more clinical data and conducting external validation in other centers to further determine its accuracy and applicability.

CONCLUSION

We developed a newly generated prognostic model that has predictive value for the prognosis of diabetic patients with AKI, which has been internally verified to have good discrimination, calibration, and clinical benefit for predicting the 90-day survival rate of patients. Future studies and external validation should validate this model in different cohorts.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MM designed the study, analyzed data, and wrote the manuscript. LP designed the study, collected and analyzed data, and wrote the manuscript. ZH designed the study and revised the manuscript. YuL collected and analyzed data. YunL and NX collected data and revised the manuscript. All authors read and approved the final manuscript.

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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.2021.737996/full#supplementary-material

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Salivary Glycopatterns as Potential Non-Invasive Biomarkers for Diagnosing and Reflecting Severity and Prognosis of Diabetic Nephropathy

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Discriminating between diabetic nephropathy (DN) and non-diabetic renal disease (NDRD) can help provide more specific treatments. However, there are no ideal biomarkers for their differentiation. Thus, the aim of this study was to identify biomarkers for diagnosing and predicting the progression of DN by investigating different salivary glycopatterns. Lectin microarrays were used to screen different glycopatterns in patients with DN or NDRD. The results were validated by lectin blotting. Logistic regression and artificial neural network analyses were used to construct diagnostic models and were validated in in another cohort. Pearson's correlation analysis, Cox regression, and Kaplan–Meier survival curves were used to analyse the correlation between lectins, and disease severity and progression. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) and bioinformatics analyses were used to identify corresponding glycoproteins and predict their function. Both the logistic regression model and the artificial neural network model achieved high diagnostic accuracy. The levels of *Aleuria aurantia* lectin (AAL), *Lycopersicon esculentum* lectin (LEL), *Lens culinaris* lectin (LCA), *Vicia villosa* lectin (VVA), and *Narcissus pseudonarcissus* lectin (NPA) were significantly correlated with the clinical and pathological parameters related to DN severity. A high level of LCA and a low level of LEL were associated with a higher risk of progression to end-stage renal disease. Glycopatterns in the saliva could be a non-invasive tool for distinguishing between DN and NDRD. The AAL, LEL, LCA, VVA, and NPA levels could reflect the severity of DN, and the LEL and LCA levels could indicate the prognosis of DN.

Keywords: saliva, glycopatterns, diagnosis, prognosis, diabetic nephropathy, non-invasive biomarkers

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INTRODUCTION

Diabetes will be the seventh leading cause of mortality by 2030, and the number of diabetes patients is expected to exceed 693 million by 2045 (1, 2). Diabetic nephropathy (DN), a serious complication of diabetes, occurs in 20% to 40% of patients with type 2 diabetes mellitus (T2DM) and is a huge economic burden on our society (3). Many scholars have found that some patients with both diabetes and kidney disease have different clinical manifestations and treatment sensitivity from typical DN patients, and this disease was coined as non-diabetic renal disease (NDRD) (4). After a kidney biopsy in patients with both diabetes and kidney disease, nearly two-thirds of patients were diagnosed with NDRD (5–7). The main types of NDRD are diabetes combined membranous nephropathy (MN), immunoglobulin A nephropathy (IgAN), and focal segmental glomerulosclerosis (FSGS) (8). DN and NDRD differ in many aspects such as pathological characteristics, clinical manifestations, treatment response, disease progression and prognosis (9). Patients with NDRD were found to have a better prognosis than patients with DN if they could receive timely treatment (10). Therefore, it is important to accurately diagnose DN and NDRD. Some doctors distinguish between DN and NDRD based on clinical experience, which may be inaccurate, leading to the risk of delaying the timing of treatment. The gold standard for the diagnosis of DN and NDRD is percutaneous renal puncture, which is a time-consuming, invasive, and expensive procedure (5). Therefore, it is of great clinical value to find a convenient and non-invasive method for differentiating DN from NDRD.

Currently, saliva is recognised as a convenient way to assess human pathological conditions, owing to its advantages in collection and storage (11, 12). Saliva is a complex oral secretion originating from the salivary gland, which is composed of many secreted proteins, electrolytes, and other substances (11). Saliva is an ideal biological fluid, which contains various substances that can reflect the health of the body (11). Salivary proteins are also widely used in the diagnosis of various diseases, such as Sjogren's syndrome, cystic fibroma, and cancer (13–16).

Lectin is a glycan-binding protein synthesised and secreted by both animal cells and plant cells. Lectin can distinguish glycopatterns according to slight structural differences and can combine with the sugar chain structure on a specific glycolipid or glycoprotein to form a covalent bond (17). Compared with antibodies, lectin costs less, is easy to obtain, and has a higher specific affinity for some glycosyl groups, which can aid in-depth analysis (18, 19). The techniques that can be used for the detection of glycoproteins include Western blotting, mass spectrometry (MS) analysis, and chromatography (20). However, these techniques have several shortcomings, such as time consumption and low efficiency, which can be circumvented using the lectin microarray technique (17). The present study used the emerging high-throughput glycosylation technology, which enabled lectin microarrays to study samples such as serum, urine, and saliva glycosylation while observing a variety of different binding reactions.

Protein glycosylation is an important and abundant post-translational modification (21). It is a process in which saccharides are transferred to the polypeptide chain skeleton, mediated by glycosyltransferase, glycosidase, and other enzymes (22). Glycosylation mainly occurs in the endoplasmic reticulum and Golgi apparatus of cells (22), and glycosylated proteins play an important role in cellular activities (22, 23).

There are two main types of glycosylated proteins in mammals: *O*-glycosylated proteins (such as mucins) and *N*-glycosylated proteins (such as erythropoietins) (24–26). Glycosylation plays an important role in the folding and conformation of stable proteins and assists a variety of biological processes through cell adhesion and recognition (27). Abnormal glycosylation is associated with many diseases, such as tumours, inflammation, and neurodegenerative diseases (28, 29). This study aimed to provide a non-invasive diagnostic tool to distinguish between DN and NDRD by analysing salivary glycopatterns and to identify biomarkers that reflect the severity and prognosis of DN.

METHODS

Recruitment Cohort

Human whole saliva was obtained from the Chinese PLA General Hospital. The study was approved by the Ethics Committee of the Chinese PLA General Hospital (No. S2014-012-01). Participants signed a written informed consent form upon collection of their saliva. This study was conducted in accordance with the Declaration of Helsinki.

Between January 2016 and October 2020, 181 eligible subjects were enrolled in this study, and the saliva of each patient was individually tested using lectin microarrays. **Figure 1** shows the design of the study. **Table S1** summarises the basic clinical characteristics of the training cohort and validation cohort. **Table S2** shows the clinical information related to diabetes. With the use of a confidence level of 0.95, a power of 0.8, a distance from mean to limits of 0.3, an SD of 0.4, and a two-sided interval, the required sample size was calculated to be 17.

The inclusion criteria were as follows: diagnosis of type 2 diabetes; pathological diagnosis of DN or NDRD; age of over 18 years; renal puncture biopsy that was performed in our hospital; and agreement to participate in the study after signing the voluntary informed consent form. The exclusion criteria were

Abbreviations: DN, diabetic nephropathy; T2DM, type 2 diabetes mellitus; NDRD, non-diabetic renal disease; MN, membranous nephropathy; IgAN, IgA nephropathy; FSGS, focal segmental glomerulosclerosis; BCA, bicinchoninic acid; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; FA, formic acid; GO, Gene Ontology; NFI, normalised fluorescence intensity; HCA, hierarchical cluster analysis; PCA, principal component analysis; ROC, receiver operating characteristic; LCA, *Lens culinaris* lectin; VVA, *Vicia villosa* lectin; NPA, *Narcissus pseudonarcissus* lectin; ACA, *Amaranthus caudatus* lectin; PHA-E+L, *Phaseolus vulgaris* lectin; EEL, *Euonymus europaeus* lectin; AAL, *Aleuria aurantia* lectin; LTL, *Lotus tetragonolobus* lectin; LEL, *Lycopersicon esculentum* lectin; DBA, *Dolichos biflorus agglutinin* lectin; PWM, *Phytolacca americana* lectin; AUC, area under the curve; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; SCr, serum creatinine; ESRD, end-stage renal disease; HR, hazard ratio; GnT-IVb, *N*-acetylglucosaminyltransferase-IVb; LacNAc, N-Acetyl-D-lactosamine; ApoA4, apolipoprotein A4.

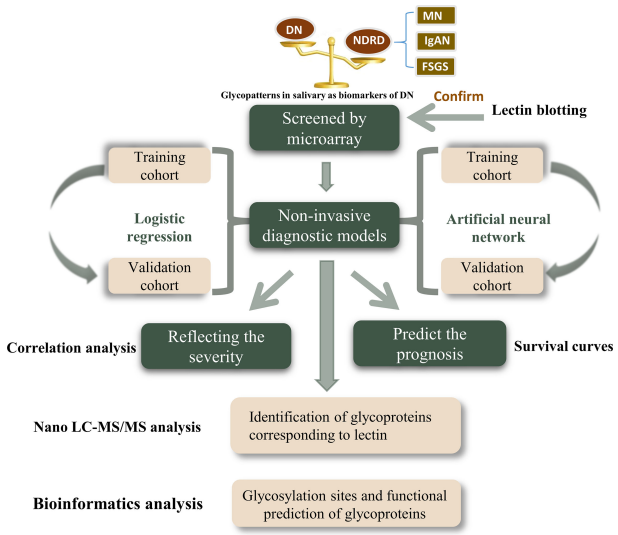

FIGURE 1 | Schematic illustration of the present research. DN, diabetic nephropathy; NDRD, non-diabetic renal disease; MN, membranous nephropathy; IgAN, immunoglobulin A nephropathy; FSGS, focal segmental glomerulosclerosis.

as follows: incomplete medical history; presence of other types of secondary renal disease such as lupus nephritis and Henoch–Schönlein purpura nephritis; patients with hereditary kidney disease; and combined urinary tract infection, malignant tumour, or pregnancy. All patients with DN and NDRD were diagnosed with pathological diagnosis through renal biopsy. The DN pathological stages were classified according to the Renal Pathology Society classification system (30). The diagnosis of NDRD followed the 2007 Kidney Disease Outcomes Quality Initiative guidelines (31). The diagnosis of the pathology was independently reviewed by two qualified pathologists.

Whole Saliva Collection

The collection methods were based on previous studies (32–34). In short, between 9 a.m. and 11 a.m., unstimulated saliva was collected at least 3 h after the last meal. Saliva samples were collected immediately after oral rinsing with sterile saline, placed on ice, and centrifuged at 10,000 × *g* at 4°C for 15 min to remove insoluble precipitates. The supernatant (1 ml) was transferred to a new tube, and 10 µl of protease cocktail inhibitor (1:100 [v/v], Sigma-Aldrich, St. Louis, MO, USA) was added. Bicinchoninic acid (BCA) assays (Beyotime Biotechnology, Shanghai, China) were used to measure the protein concentration of each saliva sample in triplicate. The treated saliva specimens were stored at −80°C until use.

Lectin Microarrays

A lectin microarray was obtained by synthesising 37 lectins with different binding preferences for the N and O chains (32). Salivary proteins were labelled with Cy3 dye (GE Healthcare, Boston, MA, USA). Cy3-labelled salivary proteins measuring 4 µg was mixed with 120 µg of incubation buffer and applied to lectin microarrays for 3 h at 37°C. Only sugar chains with a specific structure can bind to the corresponding lectin. Therefore, the fluorescence intensity of lectin represents the expression level of the corresponding glycoprotein.

Lectin Blotting

Lectin blotting was used to analyse the expression levels of the polysaccharides. Each group of concentrated salivary proteins was transferred to a polyvinylidene fluoride membrane after 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE). The membrane was then incubated with either Cy5-labelled LacNAc and poly-LacNAc conjugated with *Lycopersicon esculentum* lectin (LEL) or GalNAC terminus, GalnacαSer/Thr(TN), and GalNAcα1-3Gal conjugated with VVA.

Artificial Neural Network Prediction

An artificial neural network is a generalised model of neurobiological systems (35). Essentially, it is an attempt to simulate the human brain. Artificial neural networks can learn and replicate complex or non-linear input–output relationships by using simulated neurons. In this study, the NeuralNet Package in R (https://CRAN.R-roject.org/package=neuralnet) was used for the artificial neural network analysis. Default parameters were used, except that the argument of the hidden was fitted as H = C (30, 0).

Isolation of Glycoproteins by *Lycopersicon esculentum* Lectin-Coupled Magnetic Particle Conjugate

The proteins identified by LEL were isolated as previously described (36). Briefly, after dissolving 400 µg of LEL in 400 µl of binding solution (0.1 M of Tris-HCl, 150 mM of NaCl, 1 mM of $CaCl_2$, 1 mM of $MgCl_2$, and 1 mM of $MnCl_2$, pH 7.4), epoxysilane-coated magnetic particles (homemade) were added and incubated in binding buffer for 3 h. The 1× carbo-free blocking solution (Vector Labs, Burlingame, CA, USA) was used to block the LEL-coupled magnetic particle conjugate at room temperature for 1 h after washing three times with washing buffer (binding solution containing 0.02% Tween-20 (v/v)). Next, 1 mg of salivary protein was added to the conjugate, and the mixture was shaken and incubated to enrich glycoproteins for 3 h at room temperature. The solution was then washed thrice in washing buffer to remove non-specifically bound proteins, and the specific glycoproteins were eluted using the competitive elution buffer (100 mM of lactose). The BCA protein assay kit was used to determine the concentration of the isolated glycoproteins in triplicate.

Nano Liquid Chromatography–Tandem Mass Spectrometry Analysis

The Orbitrap Exploris 480 (Thermo Scientific, Waltham, MA, USA) equipped with an Easy n-LC 1200 HPLC system (Thermo Scientific) was used to perform all nanoscale liquid chromatography–tandem MS (LC-MS/MS) experiments. A 100

μm id × 2 cm fused silica trap column filled with reversed-phase silica gel (Reprosil-Pur C18 AQ, 5 μm, Dr Maisch GmbH, Ammerbuch, Germany) was used to load the peptides, and a 75 μm id × 20 cm C18 column filled with reversed-phase silica gel (Reprosil-Pur C18 AQ, 3 μm, Dr Maisch GmbH) was used for separation. The peptides bound to the column were eluted with a linear gradient for 73 min. Formic acid (FA; 0.1%) in water formed solvent A, and 80% acetonitrile and 0.1% FA formed solvent B. The segmented gradient was 4%–9% B, 3 min; 9%–20% B, 22 min; 20%–30% B, 20 min; 30%–40% B, 15 min; 40%–95% B, 3 min; and 95% B, 10 min, at a flow rate of 300 nl/min. Data-related acquisition mode was used to acquire MS data at a high resolution of 60,000 (m/z 200) within a mass range of 350–1,500 m/z. The target value was 3.00E+06, and the maximum injection time was 22 ms. The data-related mode was selected as the cycle time mode and set to 2 s. Precursor ions were selected from each full MS scan with an isolation width of 1.6 m/z for fragmentation in the Ion Routing Multipole with a normalised collision energy of 28%. MS/MS spectra were collected with a resolution of 15,000 at m/z 200. The target value was 7.50E+04, and the maximum injection time was 22 ms. The dynamic exclusion time was 40 s. For nano electrospray ion source setting, the spray voltage was 2.0 kV, with no sheath gas flow, and the capillary temperature was 320°C.

Database Searching and Analysis

The Proteome Discovery version 2.4.1.15 with Sequest HT search engine was used to analyse the raw LS-MS/MS data and identify the corresponding proteins. Data from saliva samples were used to search the UniProt human protein database (updated on September 2018). The proteolytic enzyme used was trypsin, and two missed cleavages were allowed. The tolerance level of the precursor was 10 ppm for MS, and the product ion tolerance was 0.02 Da for MS. Carbamidomethylation of cysteines was set as the fixed modification, and methionine oxidation was set as the variable modification. False discovery rate (FDR) analysis was performed with Percolator with the setting of FDR < 1% for protein identification. The areas of identified peptides were used for label-free protein quantification on Proteome Discovery. Only unique and razor peptides of proteins were selected for relative quantification. Normalisation mode was selected as the total peptide amount to correct for experimental bias.

Bioinformatics Analysis

The biological function and significance of proteins were obtained using Gene Ontology (GO) analysis. Blast2GO software (version 6.0) was used to characterise the biological process, molecular function, and cellular component of each protein. In addition, DAVID Bioinformatics Resources (version 6.8) was used to analyse the pathway enrichment of differential proteins. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed by mapping the thresholds of the background signal in the human genome with a count > 4 and a p-value <0.05. The STRING database was used to perform functional interaction network analysis of differential proteins. Proteins with an interaction score of 0.4 and interactions derived from text-mining were excluded.

Statistical Analysis

To reduce possible systematic variations, raw data from the lectin microarray were normalised. The median of the effective data points of each lectin was globally normalised to the sum of the median of all the effective data points in a block, which is called the normalised fluorescence intensity (NFI). The normalised data were further analysed by hierarchical cluster analysis (HCA) using Expander 6.0 (http://acgt.cs.tau.ac.il/expander/), and principal component analysis (PCA) was performed using the Multi-Variate Statistical Package (Vision 3.1 Kovach Computing Services, Wales, UK).

Normal distribution data were expressed as mean ± SD and compared using unpaired Student's t-test. Abnormal distribution data were represented as medians of the corresponding 25th and 75th percentiles (quaternary ranges) and compared using the Mann–Whitney U test. The DN diagnosis model was established according to the abundance of glycopatterns using logistic regression analysis. A receiver operating characteristic (ROC) curve was used to evaluate the diagnostic performance. Pearson's correlation was used to evaluate the correlation between lectin levels and clinical and pathological parameters related to the severity of DN. To analyse the prognosis of the DN patients, the data based on the lectin levels of patients and time to enter the dialysis phase were obtained using Cox regression, Kaplan–Meier survival curves, and the log-rank test. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed using SPSS Statistics 21.0 software (version 21.0, SPSS, Chicago, IL, USA) and GraphPad Prism software (version 8, San Diego, CA, USA).

RESULTS

Alterations of Glycopatterns Between Diabetic Nephropathy and Non-Diabetic Renal Disease

The NFIs for each lectin were summarised as the mean ± 95% CI (**Figure 2A**). The NFIs of each lectin from DN and NDRD were compared, and the results showed that the NFIs of *Lens culinaris* lectin (LCA) ($p < 0.001$), *Vicia villosa* lectin (VVA) ($p < 0.001$), *Narcissus pseudonarcissus* lectin (NPA) ($p < 0.05$), *Amaranthus caudatus* lectin (ACA) ($p < 0.01$), and *Phaseolus vulgaris* lectin (PHA-E+L) ($p < 0.05$) were significantly higher in the DN group than in the NDRD group, whereas *Euonymus europaeus* lectin (EEL) ($p < 0.05$), *Aleuria aurantia* lectin (AAL) ($p < 0.01$), *Lotus tetragonolobus* lectin (LTL) ($p < 0.05$), LEL ($p < 0.001$), *Dolichos biflorus agglutinin* lectin (DBA) ($p < 0.05$), and *Phytolacca americana* lectin (PWM) ($p < 0.05$) showed lower binding signals in the DN group than in the NDRD group. One category included the cluster of LCA and VVA, whereas the other category contained a cluster of LEL (**Figure 2B**). The LEL, VVA, and LCA with the highest significance were selected to show the recognition power for all saliva samples, depending on the results of the NFIs. Despite the small overlapping area, the LEL, VVA, and LCA could separate patients with DN from those with NDRD using PCA, as shown in **Figure 2C**. According to the results of the lectin microarray, the LEL and VVA were selected to perform lectin blotting to confirm the abundance of glycopatterns between the DN and NDRD patients.

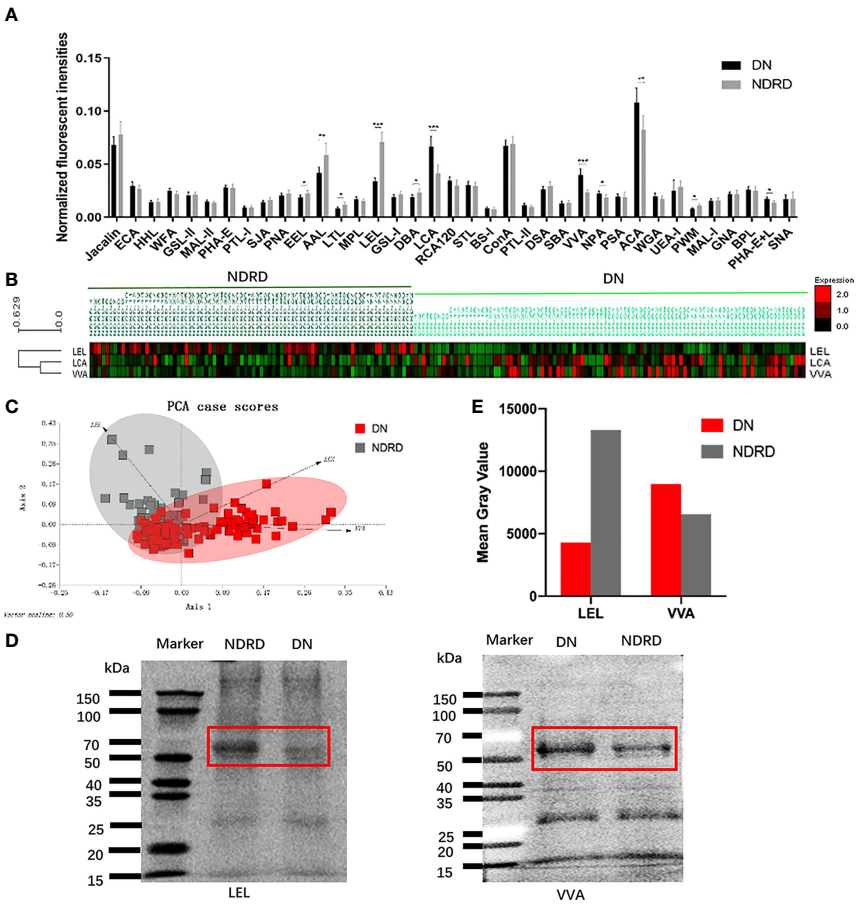

FIGURE 2 | Exhibition and confirmation of differentiation with DN and NDRD using lectins. **(A)** Comparison of all candidate lectins in salivary samples from DN and NDRD patients. The normalised fluorescence intensities of 37 lectins from DN and NDRD patients were compared based on fold-change and one-way ANOVA (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Data are presented as the mean ± 95% CI. **(B)** Hierarchical clustering analysis of the three lectins with significant differentiation of NFIs between DN and NDRD. Glycan profiles of DN and NDRD patients were clustered (average linkage, correlation similarity). Samples are listed in columns, and lectins are listed in rows. The colour intensity of each square indicates the expression levels relative to other data. Red, high; green, low; black, medium. **(C)** The normalised glycopattern abundances of three lectins related to the two groups were subjected to principal component analysis (PCA). DN and NDRD samples were visualised by red and grey shadows, respectively. **(D)** Confirmation of salivary glycopatterns from DN and NDRD groups using LEL and VVA lectins was performed by lectin blotting. **(E)** Mean grey value of each apparent difference band was obtained using ImageJ. DN, diabetic nephropathy; NDRD, non-diabetic renal disease; NFIs, normalised fluorescence intensities; LEL, *Lycopersicon esculentum* lectin; VVA, *Vicia villosa* lectin.

The red frames highlight the protein bands that showed differences between the DN and NDRD groups. The lectin blotting results show that LEL lectin specifically bound glycoproteins with a molecular weight of about 70 kDa, and their expression in patients with DN was lower than that in patients with NDRD. In contrast, VVA lectin is specifically bound to glycoproteins with a molecular weight of about 60 kDa, and their expression in patients with DN was higher than that in patients with NDRD (**Figures 2D, E**).

Establishment and Verification of the Diagnostic Model

A total of 181 participants were enrolled in this study. The baseline characteristics of the subjects are presented in **Table S1**. There were no statistical differences in the information between the training and validation cohorts, which indicates that the model is not biased. Logistic regression and artificial neural network analysis were applied to establish two diagnostic models for DN and NDRD, depending on the data of all candidate lectins in the training cohort. Diabetes-related clinical information of the DN and NDRD groups is presented in **Table S2**.

In addition, the ROC method was used to test the diagnostic models in the training and validation cohorts using the logistic regression (**Figures 3A, B**) and artificial neural network (**Figures 3C, D**) methods. We used the neural network algorithm to construct a binomial diagnosis model with 37 lectins as feature variables, and the schematic diagram of its construction process is shown in **Figure 3E**. The area under the curve (AUC) of the logistic regression model in the training and validation cohorts was 0.892 and 0.867, respectively, and the AUCs of the artificial neural network analysis model were 1.000 and 0.879, respectively. Our results showed that the models we

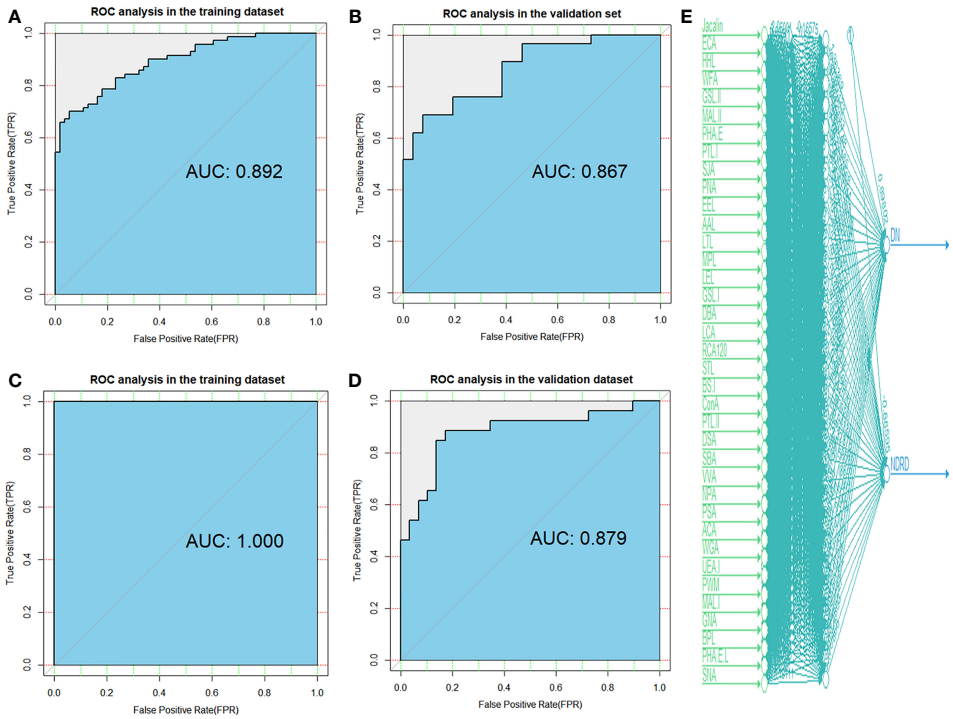

FIGURE 3 | Diagnostic accuracy of selected lectins and models was determined by ROC analysis with logistic regression and artificial neural network methods. ROC analysis for models constructed by logistic regression in the training **(A)** and validation **(B)** cohorts. ROC analysis for models constructed by artificial neural network in the training **(C)** and validation **(D)** cohorts. **(E)** A total of 37 candidate lectins for all patients are displayed in artificial neural network analysis. DN, diabetic nephropathy; NDRD, non-diabetic renal disease; ROC, receiver operating characteristic.

developed in the training cohorts also performed very well in the validation cohorts, indicating that the non-invasive diagnostic model we established has good applicability and is reliable. The parameters of the evaluation model are presented in **Table S3**.

The diagnostic model of the logistic regression is shown in Eq. 1:

$$Model\ DN = \frac{1}{1 + e^{-(2.0579 - 74.1810 * \mathrm{LEL} + 15.3656 * \mathrm{VVA} + 23.3397 * \mathrm{LCA} + 1.5249 * \mathrm{BPL})}} \quad (1)$$

Association of Glycopatterns and Severity of Diabetic Nephropathy Patients

To investigate the correlation between glycopatterns and the severity of DN, we analysed the expression levels of lectin and the clinical and pathological parameters associated with DN severity using Pearson's correlation analysis (**Table 1**). The LEL levels correlated positively with the estimated glomerular filtration rate (eGFR) ($p < 0.001$) but were negatively correlated with the blood urea nitrogen (BUN), serum creatinine (SCr), classes of glomerular lesions, and scores of interstitial and vascular lesions (all $p < 0.001$). The LCA level correlated negatively with the eGFR ($p < 0.001$) only but correlated positively with the BUN, SCr, classes of glomerular lesions, and scores of interstitial and vascular lesions (all $p < 0.001$). The VVA level correlated negatively with the eGFR ($p < 0.001$) only but correlated positively with proteinuria ($p < 0.001$), SCr ($p < 0.001$), classes of glomerular lesions ($p < 0.01$), and scores of interstitial and vascular lesions ($p < 0.001$). The AAL and NPA levels were negatively correlated with the BUN and classes of glomerular lesions (all $p < 0.05$), respectively, whereas there was no significant correlation between the EEL, LTL, DBA, ACA, PWM, BPL, and PHA-E+L levels and the clinical and pathological parameters associated with DN severity.

Association of Glycopatterns and Prognosis of Diabetic Nephropathy Patients

To investigate the association between glycopatterns and dialysis-free survival in patients with DN, multivariate Cox regression analysis was performed (**Table S4**). Higher LEL levels were associated with a reduced risk of developing end-stage renal disease (ESRD) and receiving dialysis therapy [$p < 0.001$, hazard ratio (HR) < 0.001]. However, higher LCA levels increased the risk of progressing to ESRD and receiving dialysis therapy ($p < 0.01$, HR = 730,046.848). The levels of AAL, VVA, and NPA were not significantly associated with DN progression. The Kaplan–Meier analysis was used to further investigate the relationship between the LEL and VVA levels and the dialysis-free survival of DN patients (**Figure 4**). The subjects were dichotomised based on the mean of the covariates (0.036 for LEL and 0.060 for LCA). Prolonged time to progression and

TABLE 1 | Pearson's correlation of expression levels of glycopatterns in the saliva and clinical and pathological indicators related to the severity of diabetic nephropathy.

	Proteinuria (g/24 h)	BUN (mmol/L)	eGFR (ml/min/1.73 m^2)	Scr (μmol/L)	Classes of glomerular lesions	Scores of interstitial and vascular lesions
EEL	0.049	−0.040	−0.179	0.111	0.066	0.160
AAL	−0.119	−0.215*	0.102	−0.055	0.005	−0.084
LTL	0.130	−0.085	−0.045	0.095	0.036	0.097
LEL	−0.084	−0.350***	0.884***	−0.768***	−0.739***	−0.769***
DBA	0.027	−0.038	0.009	−0.004	−0.023	0.010
LCA	0.043	0.402***	−0.757***	0.783***	0.573***	0.769***
VVA	0.606***	−0.013	−0.377***	0.450***	0.276**	0.486***
NPA	−0.038	−0.084	0.192	−0.163	−0.206*	−0.167
ACA	−0.025	−0.165	0.097	−0.030	0.023	−0.084
PWM	−0.056	0.077	0.092	−0.113	0.039	−0.138
PHA-E+L	−0.025	0.057	−0.044	0.040	0.102	0.011

The estimated glomerular filtration rate (eGFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.
BUN, blood urea nitrogen; Scr, serum creatinine; EEL, Euonymus europaeus lectin; AAL, Aleuria aurantia lectin; LTL, Lotus tetragonolobus lectin; LEL, Lycopersicon esculentum lectin; DBA, Dolichos biflorus agglutinin lectin; LCA, Lens culinaris lectin; VVA, Vicia villosa lectin; NPA, Narcissus pseudonarcissus lectin; ACA, Amaranthus caudatus lectin; PWM, Phytolacca americana lectin.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

dialysis was exhibited in DN patients with a high level of LEL, whereas a shorter time to progression and dialysis was observed in patients with a high level of LCA.

Characterisation of Protein by Liquid Chromatography–Tandem Mass Spectrometry

Based on the above results, we found that the glycoproteins that specifically bind to LEL lectin are suitable for the non-invasive diagnosis of DN. They also reflect the severity and prognosis of the disease. Therefore, we further studied the relationship between these glycoproteins and the biological processes related to DN. The proteins from DN and NDRD were respectively isolated and characterised using lectin affinity separation and LC-MS/MS. A total of 3,506 (corresponding to 740 proteins) and 3,816 (corresponding to 771 proteins) peptides were identified in DN and NDRD, respectively. Among these, the number of common peptides in both groups was 3,352 (corresponding to 720 proteins), whereas the number of proteins exclusive to DN and NDRD was 20 and 51, respectively (**Figures 5A, B**). Among the two groups of proteins identified, the relative abundance of 173 proteins between DN and NDRD changed (fold-change >2 or <0.5, $p < 0.01$) (**Figure 5C**), of which 160 proteins were significantly increased in NDRD and 13 in DN. Detailed information of the top 15 proteins with significant differences between DN and NDRD, including protein name, gene name, glycosylation site, and molecular weight information, is summarised in **Table 2**.

Bioinformatics Analysis of the Proteins Isolated From Diabetic Nephropathy and Non-Diabetic Renal Disease

To better understand the biological functions of saliva glycoproteins that specifically bind to LEL lectin in DN, GO annotations and biological function of the isolated saliva proteins from the DN and NDRD groups were obtained using Blast2GO (http://www.blast2go.org/) software. That information was classified into cellular component, biological process, and molecular function. A total of 791 proteins were identified from DN and NDRD samples, and among these, 755 proteins were annotated successfully in biological process, cellular component, and molecular function (**Figure 5D**). As shown in **Figure 5D**, in the biological process chart, 506 proteins were involved in the metabolic process, and 502 proteins were involved in the regulation of biological processes. In terms of cellular component, 370 and 357 proteins were extracellular and

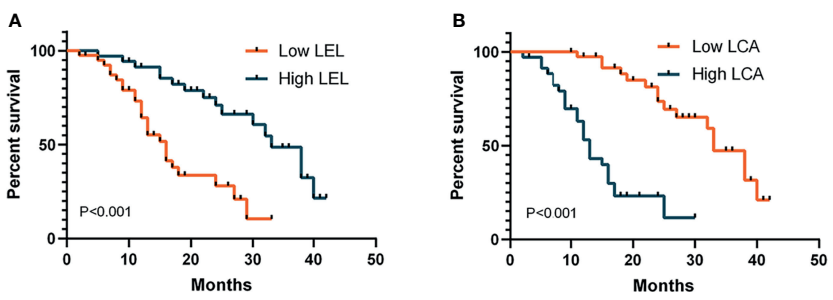

FIGURE 4 | Kaplan–Meier analysis of dialysis-free survival in patients with diabetic nephropathy. Subjects were dichotomised based on the mean of the covariates: **(A)** 0.036 for LEL; **(B)** 0.060 for LCA. p-Values refer to log-rank tests. LEL, *Lycopersicon esculentum* lectin; LCA, *Lens culinaris* lectin.

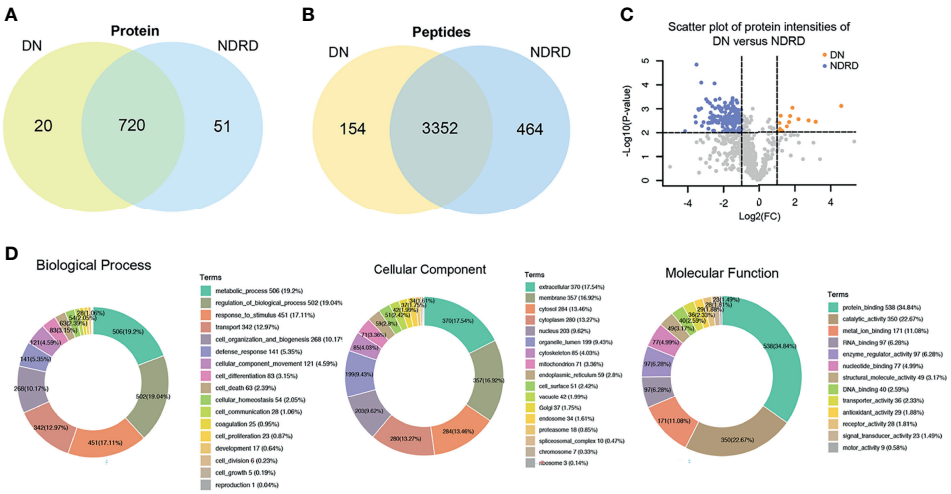

FIGURE 5 | Bioinformatics analysis of isolated glycoproteins from DN and NDRD. **(A)** Venn diagram of isolated proteins from DN and NDRD using LEL-coupled magnetic particle conjugates. **(B)** Venn diagram of isolated peptides from DN and NDRD using LEL-coupled magnetic particle conjugates. **(C)** Scatter plot of protein levels between DN and NDRD. y-Axis correspond to p-values (–log10) versus protein log2 fold-change (x-axis) in DN/NDRD. Colour indicates upregulation (orange) (fold-change > 2, $p < 0.01$) and downregulation (blue) (fold-change < 0.5, $p < 0.01$). Black represents the level of proteins without statistically significant difference between NDRD and DN. **(D)** Blast2GO was used to classify identified proteins into biological process, cellular component, and molecular function. DN, diabetic nephropathy; NDRD, non-diabetic renal disease; LEL, *Lycopersicon esculentum* lectin.

membrane proteins, respectively, and 284 proteins were cytosolic proteins. In terms of molecular function, 538 proteins with binding ability accounted for the largest proportion, and those with a smaller proportion included 350 proteins with catalytic activity and 171 proteins with metal ion binding ability. A total of 20 and 51 proteins were specially identified in the DN and NDRD groups, respectively. Thirteen proteins were significantly upregulated in DN compared to NDRD (fold-change > 2, $p < 0.01$), and 160 proteins were significantly downregulated in NDRD compared to DN (fold-change > 2, $p < 0.01$). The potential differences in GO annotations and biological function between the two groups were analysed using pathway mapping and network analysis. As shown in **Figure 6A**, the differentially expressed proteins from DN and NDRD contributed to similar biological processes, such as metabolic processes and regulation of biological processes. However, several biological processes,

TABLE 2 | Detailed information of the top 15 proteins with significant differences between the DN and NDRD groups.

Protein name	Gene	Glycosylation[a]	Mol. weight [kDa]	Fold change[b](DN/NDRD)	p-Value[c]
Haptoglobin	HP	$P^{N,O}$	45.2	0.09	<0.001
Complement C4-B	C4B; C4B_2; LOC100293534	$P^{N,O}$	192.6	0.18	<0.001
Heparin cofactor 2	SERPIND1	$P^{N,O}$	57	0.21	<0.001
Catalase	CAT	$P^{N,O}$	59.7	0.35	<0.001
Complement C3	C3	$P^{N,O}$	187	0.18	<0.001
Fibronectin	FN1	$P^{N,O}$	262.5	0.13	<0.001
Alpha-2-macroglobulin	A2M	$P^{N,O}$	163.2	0.15	<0.001
Triosephosphate isomerase	TPI1	P^{N}	30.8	0.42	<0.001
Lactoylglutathione lyase	GLO1	P^{O}	20.8	0.35	<0.001
Keratin, type I cytoskeletal 9	KRT9	$P^{N,O}$	62	23.96	<0.001
Alpha-2-macroglobulin-like protein 1	A2ML1	$P^{N,O}$	161	0.30	<0.001
Alpha-actinin-4	ACTN4	$P^{N,O}$	104.8	0.16	<0.001
Leukotriene A-4 hydrolase	LTA4H	$P^{N,O}$	69.2	0.20	<0.001
Inter-alpha-trypsin inhibitor heavy chain 4	ITIH4	$P^{N,O}$	103.8	0.37	<0.001
Alpha-actinin-1	ACTN1	$P^{N,O}$	103	0.27	<0.001

The protein expression level between NDRD and DN was compared and represented using the fold change.
DN, diabetic nephropathy; NDRD, non-diabetic renal disease.
[a]*The potential N-linked and potential O-linked glycoproteins are analysed using software NetNGlyc 1.0 and NetOGlyc 4.0 Servers and shown as "P^{N}" and "P^{O}"; protein without typical glycosylation site is shown as "N".*
[b]*The protein expression level between NDRD and DN was compared and represented using the fold change. The significant differences is setting with fold change > 2 or < 0.5 and $p < 0.001$.*
[c]*p-Value was calculated by two-tailed Student's t-test.*

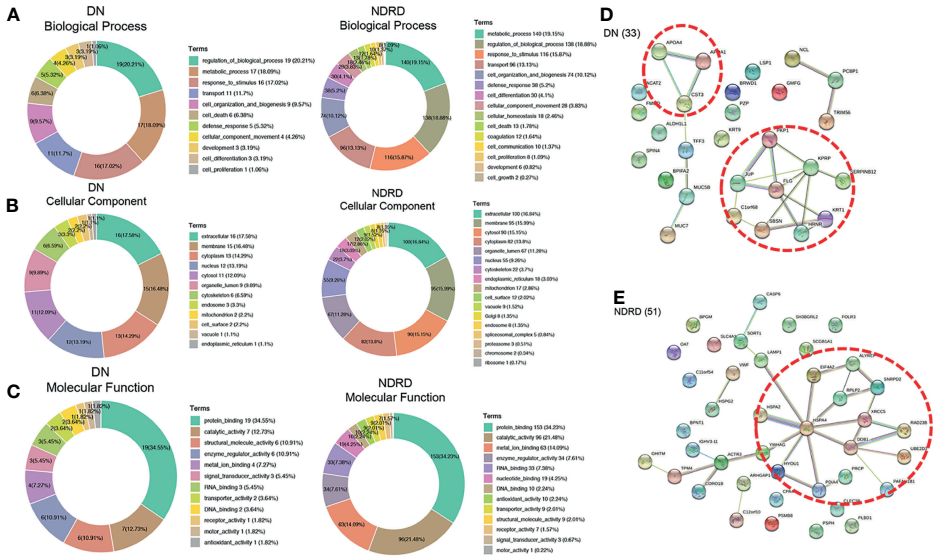

FIGURE 6 | Bioinformatics analysis of differential glycoproteins isolated from DN and NDRD. Differential proteins were analysed using Gene Ontology (GO). DN, diabetic nephropathy; NDRD, non-diabetic renal disease. **(A)** Pie charts showing the biological processes of differential proteins between DN and NDRD. **(B)** Pie charts showing the cellular component of differential proteins between DN and NDRD. **(C)** Pie charts showing the molecular function of differential proteins between DN and NDRD. Next to their position are shown the associated term names on the chart. **(D)** STRING 9.0 was used to generalise and visualise the protein interaction network of differential proteins from DN. **(E)** STRING 9.0 was used to generalise and visualise the protein interaction network of differential proteins from NDRD. Line thickness represents the strength of the association between molecules. Networks with three or more protein interactions are shown. The confidence (score) required for protein association is high. The selected protein core complexes with important functions and proteins involved in the same biochemical reaction are marked with a red dotted line.

including cellular homoeostasis, coagulation, and cell growth, were enriched in the NDRD group. In the cellular component charts, proteins related to Golgi, spliceosomal complex, proteasome, chromosome, and ribosome were only found in NDRD groups (**Figure 6B**). In the molecular function charts, the percentage of proteins with metal ion binding ability was lower in the DN group compared to the NDRD group (**Figure 6C**). Differential proteins of DN and NDRD (33 and 51, respectively) were used as defined in **Figures 6D, E**. The protein–protein interaction networks were unique in the identified proteins from the DN and NDRD groups. Two distinct protein–protein interaction sets were observed in the differential proteins of DN (**Figure 6D**), whereas one protein–protein interaction set was observed in the differential proteins of NDRD (**Figure 6E**). KEGG pathway analysis showed that the signal pathways enriched in the isolated proteins from the DN group included salivary secretion, PPAR signalling pathway, and extracellular matrix (ECM)–receptor interaction. In addition, the three most remarkable signalling pathways in the proteins from the NDRD group were complement and coagulation cascades, the pentose phosphate pathway, and the glycolysis/gluconeogenesis pathways (**Table S5**).

DISCUSSION

In recent years, diabetic kidney disease has attracted widespread attention (7, 8, 10). DN and NDRD differ in pathological characteristics, treatment response, and prognosis (37). Therefore, differentiating DN from NDRD has great clinical significance. Although renal biopsy is the gold diagnostic standard for distinguishing DN from NDRD, it is difficult to apply it to all patients because of its invasiveness and high technical proficiency required to perform the procedure. The detection of glycosylated salivary proteins by lectin has been studied in many disease fields because of its convenience, high efficiency, and accuracy.

To find an effective non-invasive diagnostic method, we used lectins to analyse the salivary glycopattern of DN and NDRD patients and to evaluate possible relationships with the severity and prognosis of DN patients. To verify the comparability between the training and validation cohorts, we compared 13 clinical indicators between the training and validation cohorts. There was no statistically significant difference in the clinical indicators between the two cohorts, which indicates that the model we built was not biased. Both models we established had their own advantages and high diagnostic accuracy for distinguishing DN from NDRD. The specificity of the logistic regression model was higher than that of the artificial neural network model in the validation cohort, whereas the sensitivity of the logistic regression model was lower than that of the artificial neural network model in the validation cohorts.

In addition, the correlation between glycopatterns and the severity and prognosis of DN was explored in this study. We analysed the correlation between the DN indicators and the 11

lectins that were differentially expressed in the DN and NDRD groups and explored whether they were related to the severity of DN. Notably, the levels of LEL, LCA, and VVA were observed to reflect the severity of DN, as revealed by Pearson's correlation analysis. The eGFR was positively correlated with the level of LEL but negatively correlated with the level of LCA and VVA, whereas the Scr, classes of glomerular lesions, and scores of interstitial and vascular lesions were negatively correlated with the level of LEL but positively correlated with the level of LCA and VVA. The BUN was negatively correlated with the LEL level but positively correlated with the LCA level. The VVA level was positively associated with proteinuria. Knowing the severity of DN aids in judging the effects of treatment and choice of the treatment plan. Therefore, the discovery of non-invasive biomarkers that can reflect disease severity is of great clinical significance. We further performed Cox regression analysis on the five lectins that can reflect the severity of DN to determine whether they are related to the prognosis of DN. Surprisingly, two lectins were found to be related to the loss of kidney function and the time to start dialysis. Low levels of LEL and high levels of LCA have been demonstrated to accelerate the deterioration to ESRD. Therefore, the LCA and LEL levels can be used as non-invasive biomarkers to assess prognosis.

Salivary glycopatterns are good indicators of health status in many diseases (33). Abnormal glucose chain structures (N-glycans) are related to the occurrence and development of tumours (38–40). Disturbance of adhesion between cells and that between cells and the ECM leads to invasion and metastasis of tumour cells (41). It has been reported that outer-arm fucosylation and core-fucosylation detected by AAL and PSA in saliva were downregulated in gastric cancer patients compared with those in healthy individuals (42–44). In a study of pancreatic cancer cell lines, it was reported that the expression of *N*-acetylglucosaminyltransferase (GnT)-IVb was mainly downregulated in adjacent tissues, and the expression of GnT-IVb was mainly upregulated in tumour tissues (45). The deterioration of inflammatory and oxidative reactions in DN patients is related to the increased expression of abnormal glycation end-product receptors on the cell surface, which leads to aberrant intracellular signal transduction and ultimately worsens the disease (46, 47). The siaα2-6Gal/GalNAc glycoprotein, which was identified by SNA, has an increased abundance in the urine of DN patients (48) The Galb1-3GalNAc glycoprotein, which was identified by BPL and has a molecular weight of approximately 53 kDa, had a decreased abundance in the serum of DN patients compared with that of NDRD patients (49). Moreover, after 5 weeks of induction, the abundance of Gal/GalNAc glycan chain structure recognised by lectin PNA and RCA dramatically declined in DN mice compared with that of control mice (50).

LC-MS/MS analysis was used to separate salivary glycoproteins containing N-Acetyl-D-lactosamine (LacNAc) identified by LEL in both the DN and NDRD groups. Twenty proteins were found only in DN patients, and 51 proteins were identified only in NDRD patients. Altered glycosylation has been shown to be a characteristic of diabetes. Elevation of serum fucose levels was observed in diabetic rat and mouse models (51, 52). It was shown that the levels of α-1,6-fucosyltransferase and glycoproteins containing fucose residues were elevated in diabetic patients (52–54). There are significant differences in glycoproteins containing fuca1–2LacNAc, biantennary complex N-glycans, a-GalNAC, Galb1–3GalNAca-Thr/Ser, and LacNAc identified using UEAI, PHA-E, GSI, PNA, and RCA in kidney glycoprotein expression between rats with or without DN (50). A previous study by our group revealed that significant differences in serum glycoproteins containing Galb1-3GalNAc and terminal GalNAc identified by BPL occur between DN and NDRD patients, and the proteins were separated using LC-MS/MS (55). Differential protein analysis showed that the expression levels of keratin type I cytoskeletal 9 were significantly higher in the NDRD group, whereas the levels of haptoglobin were significantly lower in the NDRD group. Keratin type I cytoskeletal 9 consists of a cornified envelope and participates in cell death, cell organisation, and biogenesis *via* its activity as a structural molecule. Upregulated levels of haptoglobin affect neutrophil degranulation and scavenging of heme from the plasma. The signal networks of DN patients were associated with lipid metabolism. Apolipoprotein A4 (ApoA4), a member of the Apo family, is mainly found in enterocytes in the small intestine, with a smaller amount in the liver (56). Increased levels of ApoA4 are found in the serum from DM patients (57) owing to its association with hyperglycaemia and high-density lipoprotein levels (58). In addition, the development of DM and progression to DN show a significant relationship with the elevation of ApoA4 levels (59). Therefore, ApoA4 is a potential biomarker for predicting DN.

This study has the following advantages: first, compared with previous studies that used lectin microarrays to search for biomarkers after mixing samples, we tested each patient's specimens individually on a lectin microarray for the first time. Therefore, our results are more accurate and reliable. Second, compared with previous studies that only used logistic regression to construct a diagnostic model for the results of the lectin microarray, the artificial neural network algorithm we used for the first time provided a non-invasive diagnostic model. Third, this study not only used differential lectin levels to establish a non-invasive diagnosis model for DN but also found their relationship with the severity and prognosis of DN.

This study has some limitations. First, this was a single-centre study, and multicentre and larger cohort studies are expected to further examine the diagnostic power of the model. Second, the role of the differentially expressed glycoproteins discovered in the pathogenesis of DN needs to be further explored. Third, it is advisable to extend the follow-up time to further confirm the relationship between lectin levels and the prognosis of DN.

In summary, our diagnostic models that were constructed by logistic regression and artificial neural networks could be used as non-invasive tools for distinguishing patients with DN and NDRD. The levels of AAL, LEL, LCA, VVA, and NPA could reflect DN severity, and the levels of LEL and LCA could reflect DN prognosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: ProteomeXchange, PXD030108.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Chinese PLA General Hospital (No. S2014-012-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QH and HZ contributed to the study concept. QH and XW contributed to the manuscript draft and revision. QH, HY, JH, and ZT contributed to the data analysis. QH, XW, XD, and QL contributed to supervising subject enrolment and sample collection and data collection. QH, JW, and FY contributed to performing the experiment. HZ, GC, and DZ contributed to the review and editing. HZ verified the underlying data. All authors critically reviewed and edited the manuscript and consented to final publication. HZ had full access to all the data and had final responsibility for the decision to submit for publication.

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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.2022.790586/full#supplementary-material

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