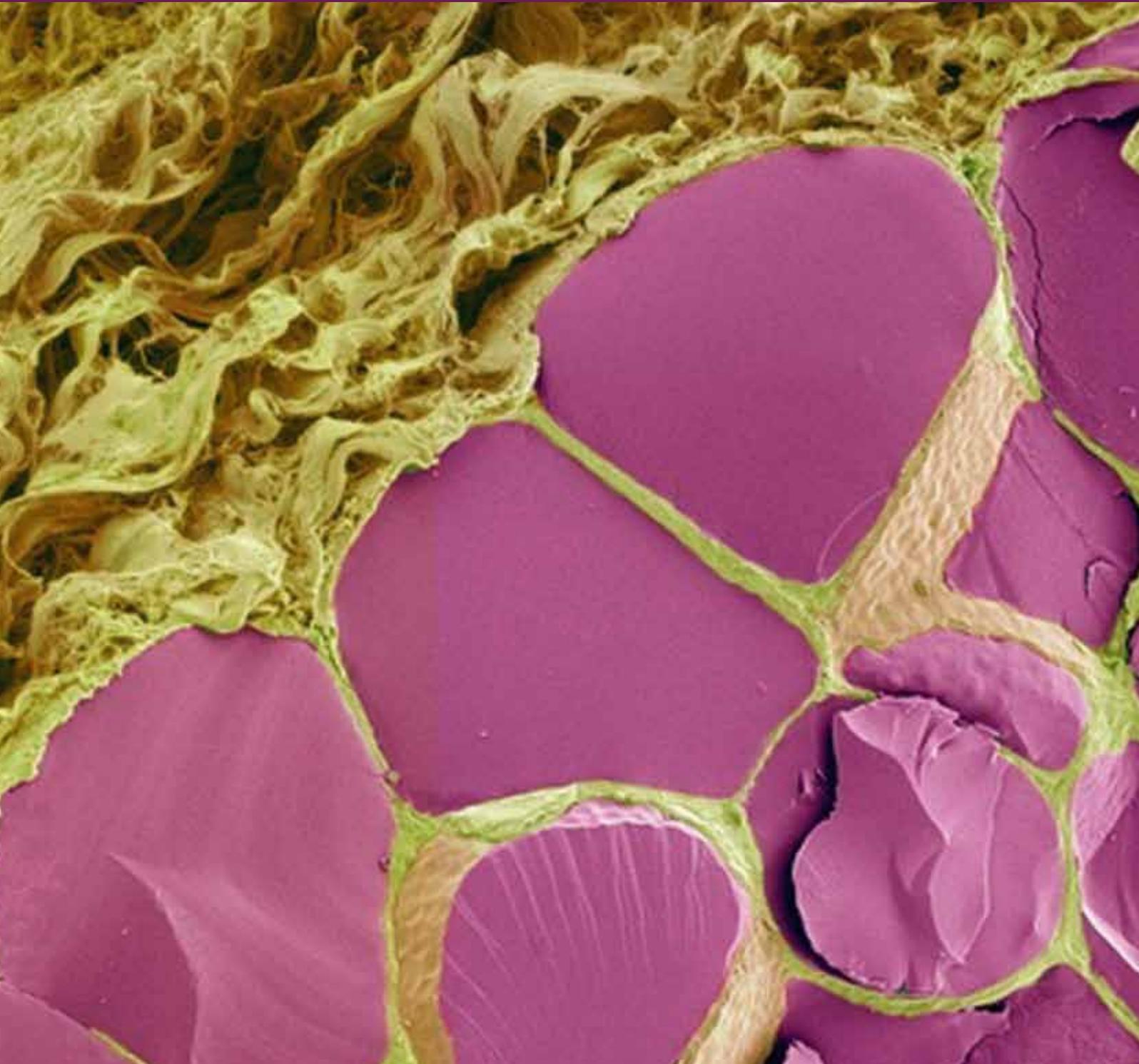


International Journal of Endocrinology

Diabetic Renal Disease

Guest Editors: Stephen Fava, Samy Hadjadj, and James Walker





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Editorial

Diabetic Renal Disease

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Diabetic nephropathy (DN) is an important long-term complication of diabetes. DN is now the most common cause of end-stage renal disease (ESRD) in many countries [1]. Both the increasing prevalence of type 2 DM and increased acceptance of diabetic patients into renal replacement therapy (RRT) programmes have contributed to this. Indeed, there has been a marked increase in the incidence of renal replacement therapy (RRT) for type 2 DM over time [1]. DN is also a major burden on health care budgets [2] and is associated with a reduction in health-related quality of life [3, 4]. Moreover, DN is associated with increased cardiovascular mortality in both type 1 and type 2 diabetic patients [5, 6].

Only a proportion of patients with DN progress to ESRD, and even in those who do progress, there is a long lag time between onset of DN and progression to ESRD. What is therefore needed is early diagnosis of DN and safe effective therapy which halts or slows down its progression as well as therapy which reduces the risk of adverse cardiovascular events in DN patients. The development of regenerative medicine for the cure of renal disease is another goal, which does not seem to be within our reach in the near future.

Diabetic nephropathy is associated with typical histological features. The Renal Pathology Society has proposed a histopathological classification of diabetic nephropathy [7]. It is hoped that this new classification might be useful in better staging of the disease and in stratifying risk. However, a renal biopsy is not indicated in the majority of cases.

The diagnosis of diabetic renal disease, therefore, usually relies on measuring the urinary albumin excretion rate and the glomerular filtration rate (GFR) and on exclusion of other causes of renal disease. Whilst an elevation in the urinary albumin excretion rate is often the earliest sign of DN, measurement of glomerular filtration is increasingly important as the disease progresses. Previously, serum creatinine alone was used as a maker of GFR. A long recognised problem of using serum creatinine as a measure of filtration function is that the GFR can fall to a clinically significant level before the creatinine level in the serum begins to rise—there being an inverse reciprocal relationship between serum creatinine and GFR. The laboratory measurement of serum creatinine using the Jaffe method has resulted in interlaboratory variability and more recently clinical chemistry laboratories have been restandardising assays against an isotope mass spectrometry method. Whilst serum creatinine levels are influenced by GFR, they are also related to muscle mass and other static and changing parameters. During the last decade many laboratories have reported estimated GFR (eGFR) using a variety of calculated formulae based on parameters including age, race, gender, creatinine (see above), and in some cases urea and albumin. The reporting of eGFRs was designed to identify those with modest degrees of renal impairment that may have been missed by clinicians relying on serum creatinine alone. The commonly used formulae for eGFRs, such as Cockcroft Gault and the Modification of Diet in

Renal Disease (MDRD) formulae, are inaccurate at GFR levels above 60 mL/min, so many laboratories will not report specific levels above 60 mL/min and will simply report a value >60 mL/min in this cohort. There are a number of circumstances where eGFR estimates are not valid including the presence of amputations, skeletal muscle diseases, extremes of age, and extremes of weight, pregnancy, and paraplegia and rapid changes in filtration function. These are often not appreciated by clinicians reviewing results on their patients. The widespread introduction of eGFR measurement has revealed many patients with eGFRs in the 30–40 mL/min range resulting in an increased rate of referral to renal departments. Whether this has resulted in clinical benefit is debatable. How much benefit have we derived from eGFR measurements?

E. Y. Lee et al. in one of the papers in this issue have compared two formulae commonly used to estimate GFR, namely, the MDRD and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) in type 2 patients with DN in Korea. They conclude that the CKD-EPI formula may more accurately stratify chronic kidney disease (CKD) in those with type 2 diabetes compared to the MDRD equation. However, this finding needs to be contrasted with the findings from the paper by X. Liu et al. which investigates the performance of the various formulas used to estimate GFR. They conclude that none of the 8 formulae examined in Chinese type 2 diabetic subjects had sufficient accuracy compared to an isotopic measurement of GFR. Inspection of the Bland Altman plots in this paper starkly demonstrates the wide levels of bias and the very wide levels of agreement between the eGFRs and standard GFR. One important point that has to be stressed is that there may be racial differences in the performance of various formulas to estimate GFR.

So what should clinicians take from these and other studies investigating eGFR equations? Firstly, they are “estimates” of the GFR in the same way that serum creatinine is an estimate and adding more parameters does not necessarily increase the accuracy. Secondly, they are likely to be inaccurate if the GFR exceeds 60 mL/min. Thirdly, there are many situations where they are likely to be particularly inaccurate and this includes using a certain formula derived from one racial group being applied in a different racial cohort.

Another area of research is the search for possible biomarkers of diabetic nephropathy. These may be useful in the diagnosis or in predicting prognosis in terms of progression or of cardiovascular complications. These include the use of proteomics, new markers of renal dysfunction, and micro-RNAs. The latter are short noncoding RNAs that may have regulatory roles. In another paper appearing in this issue, R. Li et al. review the possible role of micro-RNAs in the pathogenesis of DN and as potential biomarkers.

Although blood pressure control and blockade of the renin-angiotensin-aldosterone system are well-established as effective therapies in reducing the rate of progression of diabetic renal disease, there is still a large unmet need in the developing new treatment modalities. Research has focused on finding agents which inhibit molecules or key steps in pathways thought to be important in the pathogenesis of

diabetic nephropathy, such as the polyol pathway and transforming growth factor- β . Unfortunately, these avenues have, to date, been largely unsuccessful in providing the clinician with new therapeutic tools. In separate papers appearing in this issue, two novel therapies, namely, resveratrol (F. Xu et al.) and L-arginine (T. Claybaugh et al.), are investigated in animal models. The review paper by Li et al. appearing in this special issue also discusses the potential role of micro-RNAs as novel therapeutic modalities.

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Research Article

Modified Glomerular Filtration Rate-Estimating Equations Developed in Asiatic Population for Chinese Patients with Type 2 Diabetes

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Objectives. To evaluate eight modified equations developed in Asiatic populations in type 2 diabetic patients in China. **Methods.** A total of 209 Chinese patients with type 2 diabetes were recruited. Using the technetium—99m diethylenetriaminepentaacetic acid—glomerular filtration rate (GFR) to act as the reference, comparisons of their efficiency to estimate GFR in the subjects were made between various equations. **Results.** Median of difference of the Chinese equation 1 was the lowest (median of difference, 0.51 mL/min/1.73 m²). Median percent of absolute difference of the Chinese equation 2 was less than those of the other equations (26.97 versus ranged from 32.54 to 37.61 mL/min/1.73 m², [$P < 0.001$ for all]). Precision of the simplified reexpressed MDRD equation was the best (92.9 mL/min/1.73 m²). Accuracies of the Chinese equation 2 were greater ($P < 0.05$ for all). There was also an improvement in chronic kidney disease (CKD) stage misclassification of the Chinese equation 2 (55.0 versus ranged from 61.2 to 64.6%, [$P < 0.001$ for all]). However, the 30% accuracies of all the equations were less than 70%. **Conclusions.** Our study highlighted a limitation in the use of the above equations in the majority of Chinese diabetic subjects. A better equation is needed in order to give an accurate estimation of GFR in type 2 diabetic patients in China.

1. Introduction

Human health is confronted with increasing threat from diabetes, with the statistical data from the International Diabetes Federation displaying that there are about 285 million diabetic patients all over the world by now [1]. According to the 20th World Diabetes Congress, the population of diabetic patients in Asia will increase by 60% from 2007 to 2025 [2]. In Japan, a report in 2007 by the Ministry of Health, Labor, and Welfare showed that the estimation of the number of diabetic patients was 22 million, or a fifth of adults [3]. Based on a national survey done in 2008, the prevalence of diabetes in

China was 9.7% of the adults over the age of 20, counting for 92.4 million adults with diabetes [4].

Diabetes is associated with several complications, including nephropathy [5]. About 25–40% of diabetic patients will develop diabetic nephropathy, which is the main cause of end-stage renal disease in developed countries [6]. The estimation of kidney function is very important in diabetic subjects. Glomerular filtration rate (GFR) is the best measure of overall kidney function in health and disease [5]. GFR can be directly measured by infusion of external substances such as inulin, 51Cr-EDTA, ^{99m}Tc-diethylenetriaminepentaacetic acid (DTPA), and iohexol [7]. However, such methods

are troublesome and expensive, which limits their wide application. Therefore, a more convenient method is necessary. The National Kidney Foundation and the American Diabetes Association recommend that the modification of diet in renal disease (MDRD) equations can be used to assess GFR in adults [8, 9]. The MDRD Study equation is based on 6 variables: age, gender, ethnicity, and serum levels of creatinine, urea, and albumin [10]. Afterward, the original one was simplified to a 4-variable equation consisting of age, gender, ethnicity, and serum creatinine (SC) levels to enable its convenient clinical use [11, 12]. In 2006, the MDRD researchers used standardized serum creatinine (SC) values and developed the reexpressed MDRD equations [13]. Recently, the studies were extended to 8254 cases and the new chronic kidney disease epidemiology collaboration (CKD-EPI) equation was revised [14]. Ethnicity plays an important role in the estimation of GFR. Some researches consider that a coefficient should be used when the MDRD equations are applied to black individuals [10, 13, 15]. Taking this into account, Asiatic population should also have its own coefficient. To date, six GFR estimating equations, including the Asian equation [16], the Korean equation [17], the Japanese equation [18], the Thai equation [19], the Chinese equation 1 [20], and the Chinese equation 2 [21] were developed based on Asiatic population by amendment of the original MDRD equation. These modified equations seem more accurate in Asiatic population, but it has not been validated in diabetic patients, up until now. As diabetes is highly prevalent and costly, it is important to validate various modified equations in type 2 diabetic patients in China.

2. Materials and Methods

2.1. Sample Size. A power calculation suggested a minimum sample size of 198 using the method in Jones et al. [22]. The parameters used in the sample size formula were based on findings in a pilot study which enrolled a subgroup of patients from January 2006 to June 2008 in the same hospital (see Supplementary Table 1 and Supplementary Table 2 in the Supplementary Material available online at <http://dx.doi.org/10.1155/2014/521071>). The power of test was kept at 0.90 and level of significance at 0.01.

2.2. Subjects. A total of 209 Chinese patients with type 2 diabetes (120 males and 89 females) aged 61.6 ± 12.0 (30–89) years were enrolled consecutively from January 2005 through December 2009 in the third affiliated hospital of Sun Yat-sen University, China. Mean DTPA-GFR was 47.9 ± 26.1 (5.9–116.6) mL/min/1.73 m². Patient characteristics were depicted in Table 1. Chronic kidney disease (CKD) was diagnosed and staged based on the kidney disease: Improving Global Outcomes (KDIGO) clinical practice guidelines [23]. For convenience, stages 1 and 2 and stages 3a and 3b, as well as stages 4 and 5, were combined, respectively. Exclusion criteria include patients with acute kidney function deterioration, clinical edema, skeletal muscle atrophy, pleural effusion or ascites, malnutrition, amputation, heart failure, and ketoacidosis. Patients who were taking cimetidine or trimethoprim were excluded too. No subject was treated by dialysis at the

TABLE 1: Patient characteristics.

Sample size	209
Age (year)	61.6 ± 12.0 (30–89)
Male/female (%)	57.4/42.6
Weight (kg)	62.9 ± 11.4 (41–95)
Height (cm)	162.2 ± 8.4 (142–184)
Body surface area (m ²)	1.66 ± 0.17 (1.28–2.15)
Body mass index (kg/m ²)	23.9 ± 3.6 (16.4–38.2)
Serum creatinine (mg/dL)	2.6 ± 2.3 (0.4–10.9)
DTPA-GFR (mL/min/1.73 m ²)	47.9 ± 26.1 (5.9–116.6)
CKD stages distribution	
Stage 1	14 (6.7)
Stage 2	48 (23.0)
Stage 3a	39 (18.7)
Stage 3b	46 (22.0)
Stage 4	47 (22.5)
Stage 5	15 (7.2)

DTPA-GFR: technetium—99m diethylenetriaminepentaacetic acid—glomerular filtration rate; CKD: chronic kidney disease.

Results are expressed as mean ± SD (range) or n (%).

time of the study. The institutional review board at the third affiliated hospital of Sun Yat-sen University approved the study. Written informed consent had been obtained before the study.

2.3. Measurements of Standard GFR (sGFR). We used GFR measured by the ^{99m}Tc-DTPA renal dynamic imaging method, standardized by body surface area, as the sGFR [24, 25]. ^{99m}Tc-DTPA renal dynamic imaging (modified Gate's method) was measured by Millennium TMMPR SPECT using the General Electric Medical System. High correlation was shown in the comparison of renal dynamic imaging to inulin clearance, the reference standard for measuring GFR [26]. Renal imaging also showed good agreement with plasma clearance of ⁵¹Cr-EDTA [27]. The method of ^{99m}Tc-DTPA renal dynamic imaging was the same as previously described [28, 29].

2.4. Other Measurements. SC was determined by the enzymatic method on the Hitachi 7180 autoanalyzer (Hitachi, Tokyo, Japan; reagents from Roche Diagnostics, Mannheim, Germany) according to the manufacturer's specifications and was traceable to standard reference material (SRM 967). The following data were recorded: gender, age, height, and weight at the same time.

2.5. Estimations of GFR. The following equations were used:

- (1) Asian equation [16]: $GFR = 1.086 \times 175 \times SC^{-1.154} \times Age^{-0.203} \times (0.742 \text{ if patient is female})$
- (2) Korean equation [17]: $GFR = 87.832 \times SC^{-0.882} \times Age^{0.01} \times (0.653 \text{ if patient is female})$
- (3) Japanese equation [18]: $GFR = 194 \times SC^{-1.094} \times Age^{-0.287} \times (0.739 \text{ if patient is female})$

TABLE 2: Bias and accuracy between eGFR and sGFR.

	Median of difference (25% and 75% percentile)	Median % absolute difference (25%, 75% percentile)	Accuracy within		
			15%	30%	50%
Asian equation	1.12 (-9.36, 16.98)	33.83 (16.64, 60.59)*	21.5*	45.5*	66.0*
Korean equation	6.83 (-4.15, 22.75)*	34.38 (12.55, 52.74)*	29.7*	45.5*	72.7*
Japanese equation	-10.04 (-17.31, -0.47)*	33.71 (14.98, 52.70)*	24.9‡	45.0*	73.2*
Thai equation	2.11 (-8.68, 20.24)*	37.33 (18.66, 61.17)*	18.2*	44.0*	64.1*
Chinese equation 1	0.51 (-10.16, 22.11)‡	37.61 (19.06, 64.40)*	17.7*	38.8*	60.8*
Chinese equation 2	-1.28 (-6.88, 13.30)	26.97 (12.17, 44.80)	33.5	58.4	79.9
Simplified reexpressed MDRD equation	-2.83 (-11.34, 11.91)*	32.54 (15.72, 57.68)*	23.9*	44.5*	67.9*
CKD-EPI equation	-2.24 (-10.85, 11.82)*	32.79 (13.88, 54.49)*	26.8*	48.3*	69.4*

eGFR: estimated glomerular filtration rate; sGFR: standard glomerular filtration rate.

* $P < 0.001$ compared with Chinese equation 2-GFR.

† $P < 0.01$ compared with Chinese equation 2-GFR.

‡ $P < 0.05$ compared with Chinese equation 2-GFR.

TABLE 3: Agreement and CKD stage misclassification between eGFR and sGFR.

	Mean of difference (bias)	Precision (levels of agreement)	CKD stage misclassification
Asian equation	9.4	184.2	61.2†
Korean equation	12.3	157.4	62.7*
Japanese equation	-5.9	142.0	64.6†
Thai equation	11.6	190.0	64.6*
Chinese equation 1	12.1	202.4	64.6*
Chinese equation 2	6.7	155.9	55.0
Simplified reexpressed MDRD equation	4.9	92.9	61.7*
CKD-EPI equation	3.6	158.6	61.7*

eGFR: estimated glomerular filtration rate; sGFR: standard glomerular filtration rate; CKD: chronic kidney disease.

- (4) Thai equation [19]: $GFR = 1.129 \times 175 \times SC^{-1.154} \times Age^{-0.203} \times (0.742 \text{ if patient is female})$
- (5) Chinese equation 1 [20]: $GFR = 175 \times SC^{-1.234} \times Age^{-0.179} \times (0.79 \text{ if patient is female})$
- (6) Chinese equation 2 [21]: $GFR = 234.96 \times SC^{-0.926} \times Age^{-0.280} \times (0.828 \text{ if patient is female})$
- (7) Simplified re-expressed MDRD equation [13]: $GFR = 175 \times SC^{-1.154} \times Age^{-0.203} \times (0.742 \text{ if patient is female}) \times (1.212 \text{ if patient is black})$
- (8) CKD-EPI equation [14]: $GFR = 141 \times (SC/\kappa)^\alpha \times (0.993)^{Age} \times (1.018 \text{ if patient is female}) \times (1.159 \text{ if patient is black})$
 - (a) $\kappa = 0.7$ (female) or 0.9 (male);
 - (b) $\alpha = -0.329$ (female and $SC \leq 0.7 \text{ mg/dL}$), $\alpha = -1.209$ (female and $SC > 0.7 \text{ mg/dL}$);
 - (c) $\alpha = -0.411$ (male and $SC \leq 0.9 \text{ mg/dL}$), $\alpha = -1.209$ (male and $SC > 0.9 \text{ mg/dL}$).

2.6. Statistical Analysis. Difference between estimated GFR (eGFR) and sGFR was defined as eGFR minus sGFR. The percent of absolute difference between eGFR and sGFR was defined as the percent of absolute value of the difference. Accuracy was measured as the percentage of eGFR not

deviating more than 15%, 30%, and 50% from the sGFR. eGFR was compared with sGFR using Bland-Altman analysis. Precision was identified as the width between the 95% limits of agreement. Wilcoxon Mann-Whitney test and χ^2 test were used to compare the difference and accuracy. Prior to this study, a pilot study was conducted in a subgroup of patients selected from January 2006 to June 2008 and showed that the Chinese equation 2 performed better than the other equations (Supplementary Table 1 and Supplementary Table 2). Therefore, we chose eGFR measured by the Chinese equation 2 as the reference against which all comparisons between equations were made. All statistical analyses were performed using SPSS software (version 11.0 SPSS, Chicago, IL, USA) and MedCalc for Windows (version 9.3.9.0 MedCalc Software, Mariakerke, Belgium).

3. Results

Table 2 shows that bias of the Chinese equation 1 was the lowest (median of difference, 0.51 mL/min/1.73 m²). Median percent of absolute difference of the Chinese equation 2 was less than those of the other equations (26.97 mL/min/1.73 m² versus ranged from 31.54 to 37.61 mL/min/1.73 m², $P < 0.001$ for all). 30% to 50% accuracies of the Chinese equation 2 were greater than those of the other equations (30% accuracy, 58.4% versus ranged from 38.8 to 48.3%; 50% accuracy, 79.9%

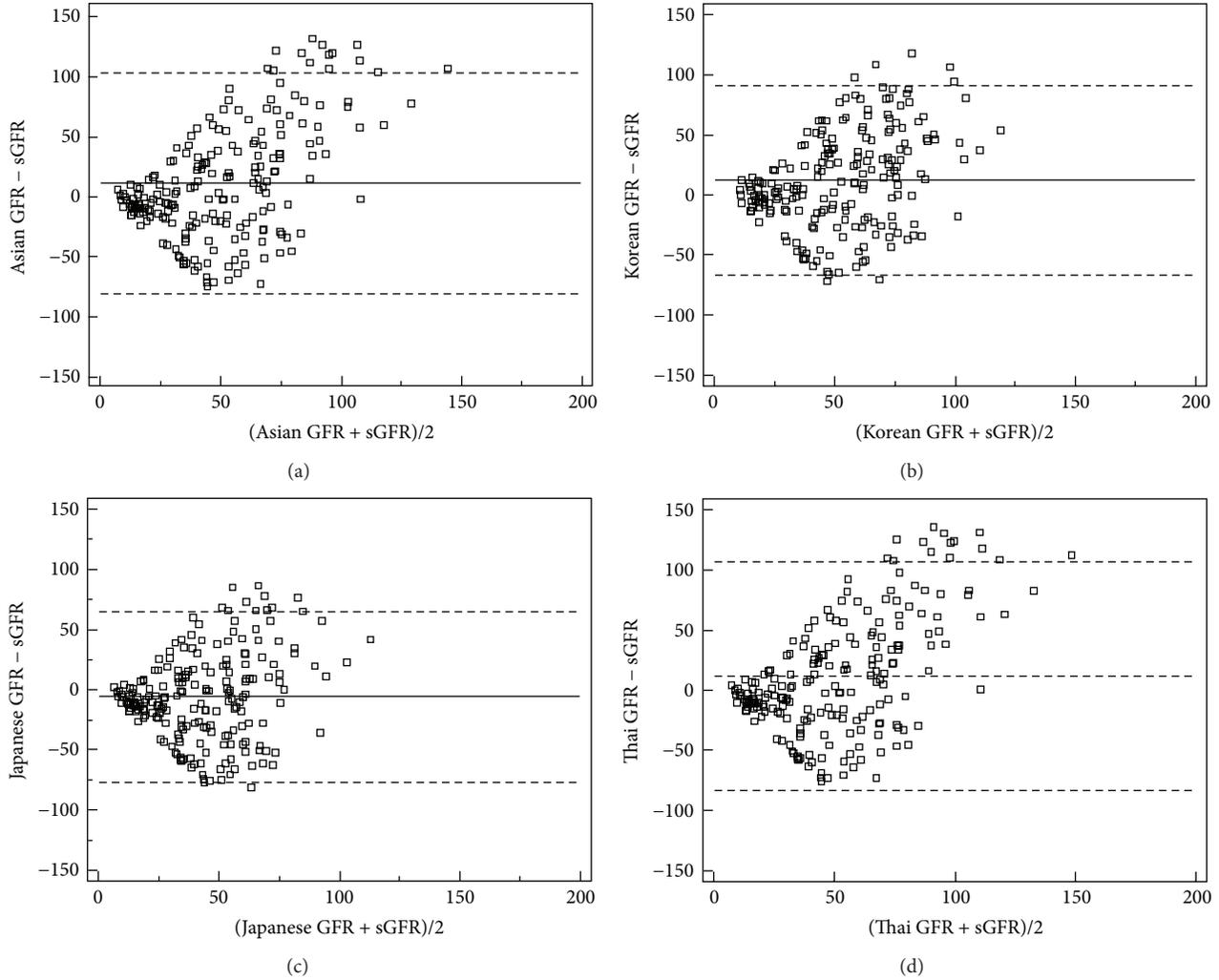


FIGURE 1: Bland-Altman plot of eGFR and sGFR ($\text{mL}/\text{min}/1.73 \text{ m}^2$)-1. Solid line represents the mean of difference between methods; dashed lines represent 95% limits of agreement of the mean of difference between methods. (a), (b), (c), and (d) represent the results of GFR estimated by Asian equation, Korean equation, Japanese equation, and Thai equation, respectively.

versus ranged from 60.8 to 73.2%, $P < 0.001$ for all), as was 15% accuracy (33.5% versus ranged from 17.7 to 29.7%, $P < 0.05$ for all). However, none of the equations had acceptable levels of 30% accuracy (at least 70%).

Table 3 and Figures 1 and 2 present that mean difference of the CKD-EPI equation ($3.6 \text{ mL}/\text{min}/1.73 \text{ m}^2$) and precision of the simplified reexpressed MDRD equation ($92.9 \text{ mL}/\text{min}/1.73 \text{ m}^2$) were the best. There was an improvement in CKD stage misclassification of the Chinese equation 2 (55.0% versus ranged from 61.2 to 64.6%, $P < 0.001$ for all). And the CKD stage misclassification of all the equations exceeded 54%.

Table 4 shows the performance of the eight equations in various stages of CKD. In CKD stages 1 and 2, median of difference of the Japanese equation was the least. Both median percent of absolute difference and accuracies of the CKD-EPI equation were better than those of the other equations. In CKD stages 3a and 3b, median of difference of the CKD-EPI

equation was less than those of the other equations. In CKD stages 4 and 5, the Korean equation displayed less median of difference. The Chinese equation 2 yielded improved median percent of absolute difference and accuracies in CKD stages 3a-3b and CKD stages 4-5, as well as the CKD stage misclassification in all CKD subgroups. The performances of all the equations were progressively deteriorating with declining CKD stage.

4. Discussion

Diabetes is the primary cause of CKD in the USA [30]. A research by Rigalleau et al. showed that the MDRD equation was more accurate for the diagnosis and stratification of renal failure in diabetic patients [31]. The abbreviated MDRD equation [10] has been the most widely used in clinical practice, becoming a powerful screening tool for early detection of CKD. However, consensus on the most

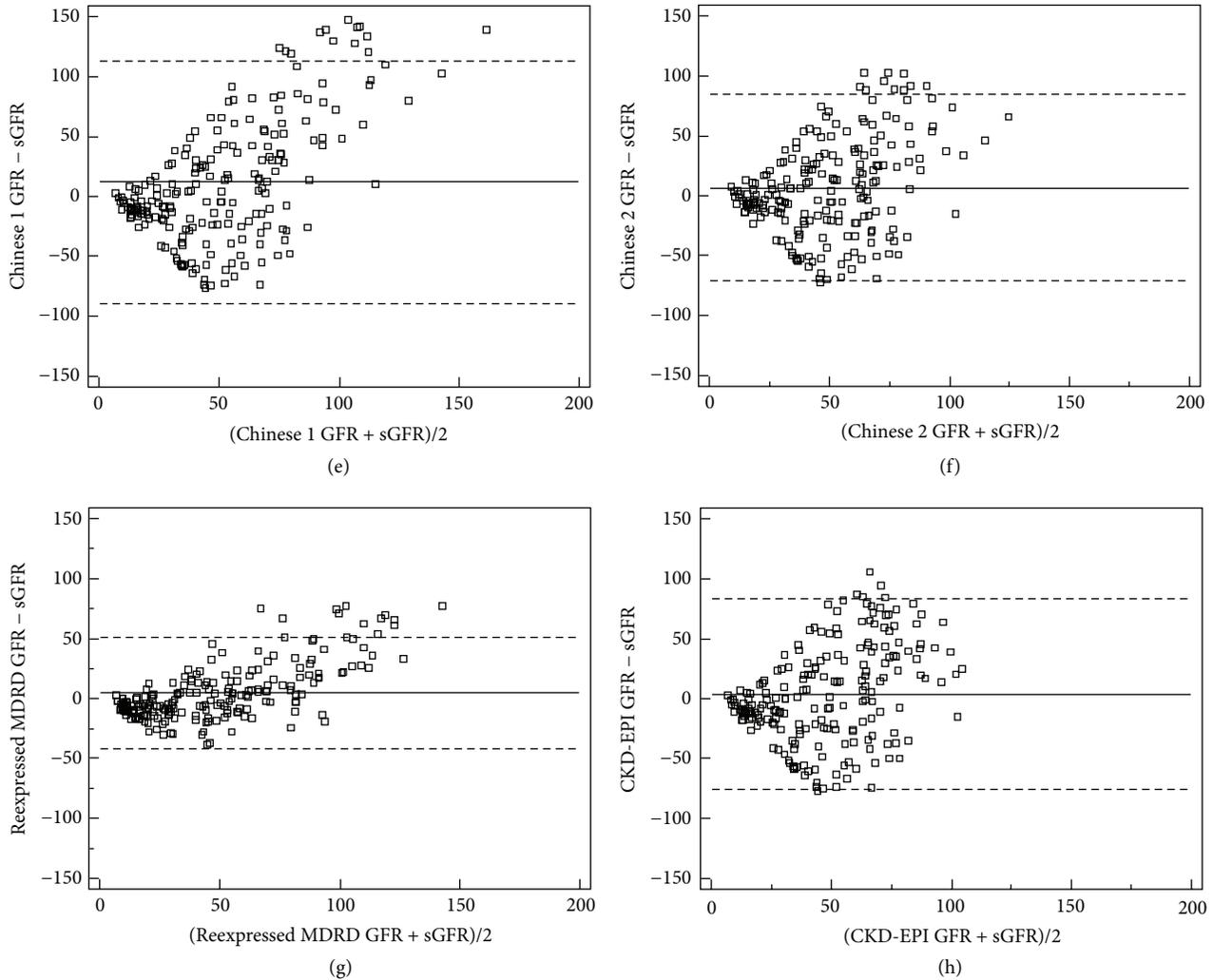


FIGURE 2: Bland-Altman plot of eGFR and sGFR ($\text{mL}/\text{min}/1.73 \text{ m}^2$)-2. Solid blue represents the mean of difference between methods; dashed lines represent 95% limits of agreement of the mean of difference between methods. (e), (f), (g), and (h) represent the results of GFR estimated by Chinese equation 1, Chinese equation 2, Simplified reexpressed MDRD equation, and CKD-EPI equation, respectively.

appropriate equation for Chinese diabetic patients has not got, and researches in this respect are very limited. Therefore, it is essential to undertake a study on this issue. In our study, we made comparisons between all the six modified equations developed in Asiatic population as well as the simplified reexpressed MDRD equation and the CKD-EPI equation, aiming to find out a better predictor of GFR for Chinese type 2 diabetic patients. In both the overall result and the results in different stages of CKD, GFR estimated by the Chinese equation 2 achieved the best performance. However, none of the equations had acceptable levels of 30% accuracy (at least 70%), which implied that a more accurate equation was needed to give a better prediction for Chinese type 2 diabetic patients.

So why did these equations fail to apply in type 2 diabetic patients in China and where did the bias come from?

The population studied was different. In our study, the subjects were type 2 diabetic patients in China. However, all the modified equations [16–21] as well as the simplified

reexpressed MDRD equation [13] and the CKD-EPI equation [14] used to estimate GFR were established in CKD patients instead of diabetic patients, which imposed restrictions on the application of the equations. And patients studied by the Asian equation, the Korean equation, the Japanese equation, the Thai equation, the Chinese equation 1, the Chinese equation 2, and the simplified reexpressed MDRD equation were all a small part of the large population [32]. The pooled data sets in the CKD-EPI equation across various study populations and clinical conditions, which allows more general applicability than does the other equations [14]. And some differences in the performance of GFR predicting equations between various CKD as well as age subgroups were found in this study. Besides the above problems debated, ethnicity is another factor for the bias [19], which can influence the applicant of estimated equations.

The methods used to measure sGFR were different. Both in Korea and Japan, renal inulin clearance was used as the sGFR [17, 18], which was different to the method (DTPA renal

TABLE 4: Performance between eGFR and sGFR in different subgroups of CKD.

	Median of difference (25% and 75% percentile)	Accuracy within		CKD stage misclassification	
		15%	30%		50%
Performance in CKD stages 1-2 (n = 62)					
Asian equation	20.63 (-1.57, 48.63)* [@]	27.4*	43.5*	64.5*	56.5 [#]
Korean equation	18.91 (-1.67, 30.96) [†] &	37.1 [†]	51.6*	87.1 [#]	53.2* ^{&}
Japanese equation	-7.25 (-22.04, 9.93)*	32.3	66.1 [†]	87.1 [†]	54.8 [#]
Thai equation	12.45 (1.06, 53.80) [†] @	21.0*	41.9*	59.7*	61.3 [#]
Chinese equation 1	27.43 (2.89, 63.99) [†] @	17.7 [†]	38.7*	56.5*	61.3 [#]
Chinese equation 2	9.72 (-4.71, 29.98) [#]	40.3	59.7	85.5	53.2
Simplified reexpressed MDRD equation	13.01 (-6.85, 38.11)* ^{&}	30.6*	53.2*	75.8*	56.5*
CKD-EPI equation	10.06 (-4.94, 26.66) ^{&}	40.3*	64.5*	88.7* ^{&}	54.8 [#]
Performance in CKD stages 3a-3b (n = 85)					
Asian equation	1.72 (-9.92, 14.04)	23.5 [†]	52.9 [†]	71.8 [†]	74.1 [†]
Korean equation	10.72 (-3.64, 23.35)*	25.9	42.4*	69.4	75.3
Japanese equation	-12.02 (-21.52, -2.49)*	29.4	47.1 [†]	77.6	72.9
Thai equation	3.99 (-8.83, 16.10) [†]	23.5 [†]	50.6 [†]	69.4 [†]	78.8 [†]
Chinese equation 1	0.98 (-11.56, 14.97)	20.0 [†]	47.1*	70.6 [†]	76.5 [†]
Chinese equation 2	1.28 (-8.90, 12.10)	34.1	62.4	81.2	65.9
Simplified reexpressed MDRD equation	-2.42 (-12.90, 9.27)*	27.1	54.1	74.1 [†]	71.8
CKD-EPI equation	-0.54 (-12.53, 11.77)*	27.1	55.3	70.6 [†]	71.8
Performance in CKD stages 4-5 (n = 62)					
Asian equation	-5.87 (-10.49, 1.04) ^{**}	12.9	37.1 [†]	59.7*	48.4* ^{&}
Korean equation	0.93 (-5.03, 10.03) ^{**}	27.4	43.5*	62.9*	54.8 [#]
Japanese equation	-8.22 (-12.54, -3.35)*	11.3 ^{&}	21.0 ^{&}	53.2 ^{&}	62.9*
Thai equation	-5.56 (-10.26, 1.62) ^{**}	8.1 [†]	37.1*	61.4*	51.6* [@]
Chinese equation 1	-6.41 (-11.55, -0.47) ^{**}	14.5	27.4 [†]	51.6 [†]	51.6* ^{&}
Chinese equation 2	-1.95 (-6.94, 5.16)	25.8	51.6	72.6	41.9 ^{&}
Simplified reexpressed MDRD equation	-6.48 (-11.30, -0.21)*	12.9 ^{&}	22.6 [@]	51.6 ^{&}	53.2 [#]
CKD-EPI equation	-6.90 (-11.56, -0.47)*	12.9 [#]	22.6 [@]	48.4 [†] &	54.8 [#]

eGFR: estimated glomerular filtration rate; sGFR: standard glomerular filtration rate; CKD: chronic kidney disease.

*P < 0.001 compared with Chinese equation 2-GFR.

†P < 0.01 compared with Chinese equation 2-GFR.

‡P < 0.05 compared with Chinese equation 2-GFR.

@P < 0.001 compared with the subgroup with CKD stages 3a-3b.

&P < 0.01 compared with the subgroup with CKD stages 3a-3b.

#P < 0.05 compared with the subgroup with CKD stages 3a-3b.

dynamic imaging) used in the Chinese equation 2 [21] as well as our study and the plasma clearance of DTPA used in the Asian equation [16], the Thai equation [15], and the Chinese equation 1 [20]. Urinary clearance of ^{125}I -iothalamate was used as the sGFR in the reexpressed MDRD equation [13] and the CKD-EPI equation [14]. According to a research in 2011, underestimation of sGFR by plasma clearance of DTPA while overestimation by DTPA renal dynamic imaging were found in comparison with the inulin clearance method [26]. These could bring about bias in the estimations of sGFR in diabetic patients in China.

The calibrations of SC were different. SC levels in the Asian equation [16], the Korean equation [17], the Thai equation [19], the reexpressed MDRD equation [13], the CKD-EPI equation [14], and our study were all calibrated to an assay traceable to isotope-dilution mass spectrometry. Creatinine value was obtained by the enzyme method for the Japanese equation [18], which was calibrated to the noncompensated Jaffé method in the Cleveland Clinic laboratory in 1990. In the Chinese equation 1 [20], the SC value, which was measured by the Jaffé's kinetic method, was calibrated to the SC value measured by the Cleveland Clinic Laboratory by using a CX3 analyzer. In the Chinese equation 2 [17], the SC value was also measured by the Jaffé's kinetic method. Different ways to calibrate the data could lead to inaccuracy in equation. Variability among laboratories in the calibration of SC measurement was of critical importance in GFR estimation.

A new equation was needed to give an exact prediction of GFR in type 2 diabetic patients in China. We may take the issue discussed below into considerations.

This study displayed that the ethnicity coefficients developed in these studies might not be adequate for the management of Chinese diabetic patients, due to the difference in the calibration of SC and GFR measurement protocol and the inclusion criteria of patients. For better comparisons of different methods to estimate GFR, we had better standardize the methods to determine the value of SC and sGFR and the same inclusion criterion.

Characteristics of diabetes should be considered. None of the GFR estimated equations were based on the human physiological mechanism. They were gotten through the statistical analysis software by analyzing the data from demography. Diabetic patients were different from the ordinary CKD patients, other parameters such as the course of disease, blood-glucose level, and albuminuria, which could affect the progression of renal impairment, might be also included in the estimations of GFR. Related investigations found that many diabetic patients had a supernormal GFR before the onset of overt clinical diabetic nephropathy and progressive renal insufficiency [33–35], and the subsequent course in these patients implied that such homodynamic abnormalities may herald the development of diabetic nephropathy [36]. But study showed that MDRD was underestimated when the GFR was above or near the normal GFR [37]. We failed to find these patients, whose renal function could be restored if intervention measures were taken timely, until now. So the early detection of the supernormal GFR should not be neglected by the predicted equations.

5. Limitations

We had incomplete data on glycaemic status that might alter the estimation of GFR [38].

6. Conclusions

Our findings highlighted a limitation in the use of all the six modified equations developed in Asiatic population, as well as the simplified reexpressed MDRD equation and the CKD-EPI equation in diabetic subjects. A better equation is needed in order to give an accurate estimation of GFR for Chinese type 2 diabetic patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Contribution of each author: Xun Liu, Jianhua Huang, Chenggang Shi, and Tanqi Lou planned the project; Xun Liu and Xilian Qiu carried out the experimental work; Xun Liu, Hui Huang, and Ming Li intellectually analyzed the data; Xun Liu wrote the paper. Xun Liu and Xilian Qiu contributed equally to the paper.

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Research Article

Resveratrol Prevention of Diabetic Nephropathy Is Associated with the Suppression of Renal Inflammation and Mesangial Cell Proliferation: Possible Roles of Akt/NF- κ B Pathway

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The present study was to investigate the protection of resveratrol (RSV) in diabetes associated with kidney inflammation and cell proliferation. Rat mesangial cell and streptozotocin-induced type 1 diabetes mouse model were used. *In vitro*, RSV attenuated high glucose-induced plasminogen activator inhibitor (PAI-1) expression and mesangial cell proliferation, as well as Akt and nuclear factor-kappa B (NF- κ B) activation. The similar results were recaptured in the experiment with Akt inhibitors. *In vivo*, mice were divided into three groups: control group, diabetes mellitus (DM) group, and RSV-treated DM group. Compared with control group, the kidney weight to body weight ratio and albumin to creatinine ratio were increased in DM group, but not in RSV-treated DM group. Furthermore, the increased expression of PAI-1 and intercellular adhesion molecule-1 in diabetic renal cortex were also reduced by RSV administration. Besides, the kidney p-Akt/Akt ratio and NF- κ B were significantly increased in DM group; however, these changes were reversed in RSV-treated DM group. Additionally, immunohistochemistry results indicated that RSV treatment reduced the density of proliferating cell nuclear antigen-positive cells significantly in glomeruli of diabetic mice. These results suggest that RSV prevents diabetes-induced renal inflammation and mesangial cell proliferation possibly through Akt/NF- κ B pathway inhibition.

1. Introduction

Nowadays, diabetic nephropathy (DN) has become a serious problem worldwide because of its rapidly increasing rates, as well as economic and social burden. Unfortunately, the intimate mechanisms leading to the development and progression of this disease are complex and not yet fully understood [1].

Glomerular mesangium expansion is one of the characters of early DN. Accumulated data suggest that the predicted evolution of diabetic glomerulopathy is comprised of an early, transient mesangial cell proliferation and subsequent hypertrophy of these cells that herald the slow progression into glomerulosclerosis [2]. In addition, inflammation is also an important pathophysiological factor in the development and progression of DN [3, 4]. Recent studies have emphasized

the critical roles of inflammatory response in development of DN [5, 6]. Different inflammatory molecules, including chemokines, adhesion molecules, and proinflammatory cytokines, may be critical factors involved in DN.

Resveratrol (RSV) is a phytoalexin polyphenolic compound found in various plants, such as grapes, nuts, and berries. What is more, the number of plants involving this compound is growing [7]. A series of potential beneficial effects of RSV should be attributed to its multiple bioactivities. Function of RSV has been extensively explored for its powerful antioxidant capacity and specific effects on proteins and/or signaling cascades, such as Sirt1, adenosine monophosphate activated kinase, phosphatidylinositol-3 kinase (PI3K)/Akt, and JNK/nuclear factor-kappa B (NF- κ B) in DN both *in vivo* and *in vitro* [8–10]. By using 12-week old *db/db* mice, Kim et al. found that RSV decreased the

activity of PI3K/Akt phosphorylation, resulting in a decrease in BCL-2-associated X protein (BAX) and increases in BCL-2 and superoxide dismutase production in diabetic kidney [8]. Additionally, Zhang et al. demonstrated that RSV prevented high glucose-induced kidney mesangial cell proliferation and fibronectin expression through inhibition of high glucose-induced JNK and NF- κ B activation, NADPH oxidase activity elevation, and reactive oxygen species production [10]. However, whether there is a direct link between Akt and NF- κ B for the protection of RSV from DN was not addressed in these two separate papers. In the present study, therefore, we aimed to determine whether RSV treatment attenuated renal inflammation and mesangial cell proliferation under diabetic condition both *in vivo* and *in vitro*. By using Akt activity inhibitors, we have mechanistically defined whether the protective effect of RSV on DN was due to Akt-dependent depression of NF- κ B.

2. Research Design and Methods

2.1. Rat Mesangial Cell (RMC) Culture and Treatment. RMCs were cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Scientific Hyclone, Beijing, China) containing 5.6 mM glucose (normal glucose, NG), 10% Fetal Bovine Serum (FBS, Thermo Scientific Hyclone, Beijing, China), 100 U/mL penicillin (Thermo Scientific Hyclone, Beijing, China), and 100 μ g/mL streptomycin (Thermo Scientific Hyclone, Beijing, China). RMCs were exposed to 25 mM D-glucose (high glucose, HG) with 0.2% bovine serum albumin (BSA) and 0.5% FBS for 10 min–48 h. D-mannitol (19.5 mM) was used as a hyperosmotic control. LY294002 (LY, 10 μ M, Sigma-Aldrich Co., St. Louis, MO, USA), MK-2206 (MK, 1 μ M, Selleck Chemicals Co., Houston, TX, USA), or RSV (25 μ M, Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO) was added. Cells were harvested at the indicated times. Akt (Cell Signaling Technology, Danvers, MA, USA), phospho-Akt (p-Akt, Ser473, Cell Signaling Technology, Danvers, MA, USA), NF- κ B p65 (Cell Signaling Technology, Danvers, MA, USA), plasminogen activator inhibitor (PAI-1, Abcam Inc., Cambridge, MA, USA), and β -Actin (Cell Signaling Technology, Danvers, MA, USA) expression were determined by Western blotting assay.

2.2. Cell Proliferation Assay. Cells were seeded into 96-well plates at a proper density. When the confluence reached at 60%–70%, the medium was replaced with DMEM containing NG (5.6 mM) and 0.2% BSA. 24 h later, the cells were pretreated with 10 μ M LY294002, 1 μ M MK-2206, or an equal volume of DMSO for 30 min and then incubated for another 24 h with or without HG (25 mM) in the presence or absence of RSV (25 μ M). Cell proliferation was determined by Cell Counting Kit-8 (CCK8, Beyotime, Shanghai, China) or by bromodeoxyuridine (BrdU) incorporation using the cell proliferation ELISA kit (Roche, Mannheim, Germany) according to manufacturer's procedures.

2.3. Experimental Animals. Male FVB mice at eight weeks of age and 26–30 g weight, were purchased from Vital River

Laboratory Animal Technology Co. Ltd. and housed in Jilin University Animal Center under standard vivarium conditions (22°C, 12 h light/dark cycle) with free access to water and standard rodent chow. The animals were acclimatized to the laboratory conditions for 2 weeks prior to the inception of experiments. All animal procedures were approved by the University Animal Care and Use Committee, which is certified by the Chinese Association of Accreditation of Laboratory Animal Care.

2.4. Induction of Experimental Models. Mice were randomly divided into three groups (each group contains at least 6 mice): control group, diabetes mellitus (DM) group, and RSV-treated DM group. Experimental diabetes was induced with multiple low doses of streptozotocin (STZ). Mice were injected intraperitoneally with STZ (Sigma-Aldrich, St. Louis, MO, USA), which was freshly dissolved in cold citrate buffer (pH 4.5), at a concentration of 50 mg/kg daily for 5 consecutive days. And control mice received multiple injections of the same volume of sodium citrate buffer. Five days after the last injection, mice with moderate diabetes (i.e., blood glucose concentration \geq 14 mM, 3 consecutive days) were selected for the experiment. RSV-treated DM group was given RSV by oral gavage in a dose of 10 mg/kg/day for 12 weeks. At the same time, both control and DM groups were given an equivalent amount of saline by oral gavage for the same period. The dosage was adjusted every week based on any change in body weight during the whole period of study. After 12-week treatment with RSV or saline, the mice were fasted overnight, anaesthetized, and killed by cervical decapitation.

2.5. Mouse Urinary Albumin to Creatinine Ratio (ACR) Detection. Spot urine was collected before mice were killed. Urinary albumin and creatinine excretion were determined using Mouse Albumin ELISA Kit (Bethyl Laboratories Inc., Montgomery, TX, USA) and QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's procedures. Mouse urinary ACR was calculated as $ACR = \text{urinary albumin/urinary creatinine } (\mu\text{g/mg})$ as we described before [11].

2.6. Kidney Histology and Immunohistochemistry. The kidneys were harvested and fixed in 10% formalin. 5 μ m thick sections were stained with periodic acid-Schiff (PAS) reagent. Immunohistochemistry was performed in paraffin sections using a high-temperature-heating antigen retrieval method. Primary antibody used in the present study was proliferating cell nuclear antigen (PCNA, Maixin, Fuzhou, China). After being incubated with the secondary antibody (Proteintech Group, Chicago, IL, USA), 2 μ m thick sections were developed with SP immunohistochemical kit (Maixin, Fuzhou, China) to produce a brown product and counterstained with hematoxylin. Histologic evaluation was performed using a Nikon Eclipse E600 microscopy system without knowledge of the identity of the various groups.

2.7. Real-Time PCR. Total RNA of kidney samples was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA),

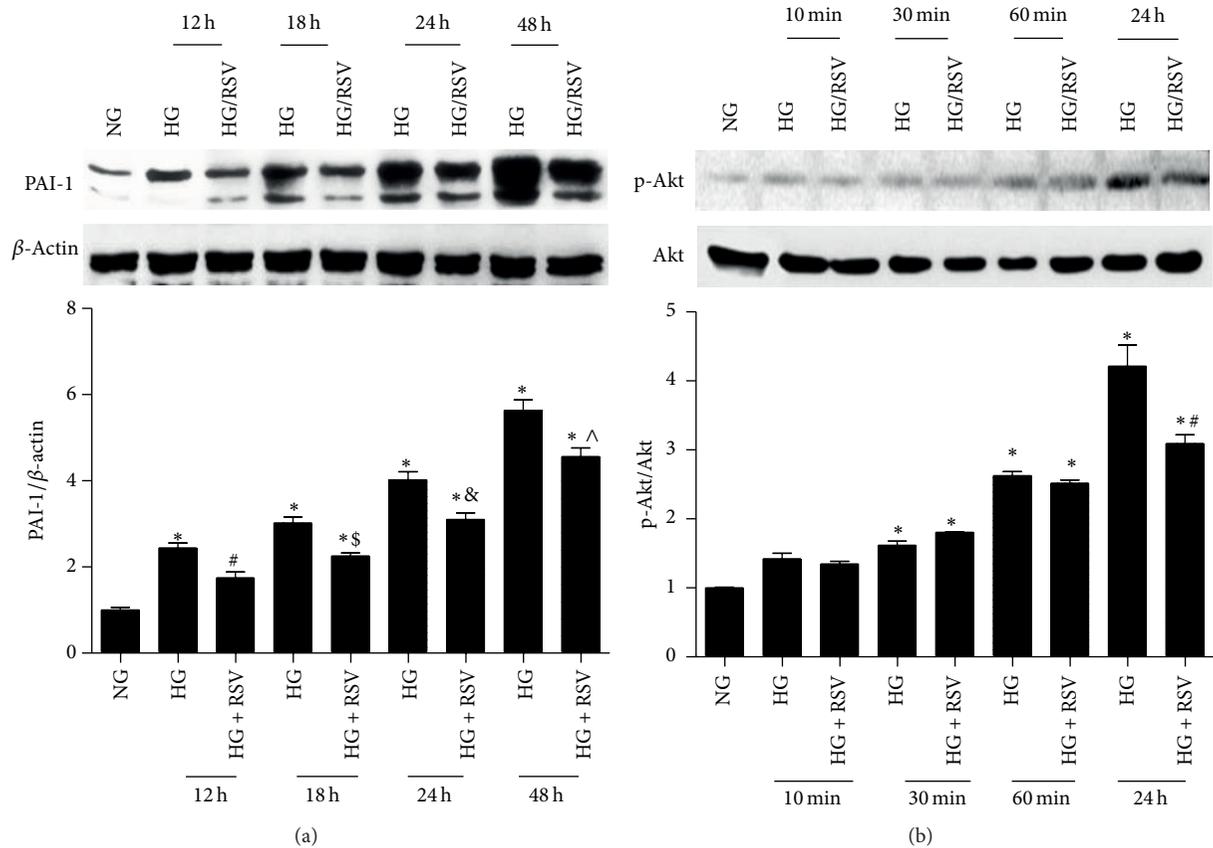


FIGURE 1: Resveratrol (RSV) attenuated HG-induced PAI-1 expression and Akt activation in rat mesangial cell (RMC). RMCs were cultured in DMEM containing 5.6 mM glucose (NG) or 25 mM glucose (HG) in the absence or presence of RSV (25 μ M) for different periods of time, the protein levels of plasminogen activator inhibitor (PAI-1) (a); Akt and p-Akt (b) were detected using Western blotting assay. Results represent the mean \pm SD. * P < 0.05 compared with NG, # P , \$ P , & P , and ^ P < 0.05 compared with HG in the same time group.

according to the manufacturer's instructions. RNA concentrations and purities were quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was reverse-transcribed from total RNA with reverse transcription-PCR kit (Takara, Shiga, Japan), according to manufacturer's protocol. Real-time PCR was carried out in the ABI 7300 Real-time PCR system to determine the change in expression of various mRNA levels as described before [12]. The housekeeping gene GAPDH was used as an internal control.

2.8. Western Blotting Assay. The kidney tissues were homogenized and the cells were sonicated in RIPA buffer (Solarbio, Shanghai, China). The proteins were electrophoresed on 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk for 1 h and then were incubated overnight at 4°C with the following primary antibodies: PAI-1, intercellular adhesion molecule-1 (ICAM-1, Abcam Inc., Cambridge, MA, USA), Akt, p-Akt, NF- κ B, and β -Actin. After four times washing with TBST, membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. Immunoreactive bands were developed by enhanced chemiluminescence after triple washing with PBS-Tween and scanned by an automatic

digital gel image analysis system (Tanon-4200, Tanon, Shanghai, China).

2.9. Statistical Analysis. *In vivo* and *in vitro* data were collected from at least six animals or at least three separate cell cultures for each group and presented as means \pm SD. Comparisons between groups were performed by one-way ANOVA, followed by Tukey's post hoc test. Statistical analysis was performed with Prism 6.0 data analysis and graphing software. Statistical significance was considered as P < 0.05.

3. Results

3.1. RSV Attenuated HG-Induced PAI-1 Expression and Akt Activation In Vitro. Quiescent RMCs exposed to HG (25 mM) for indicated time were treated with or without RSV (25 μ M). After that, cells were harvested for analysis. As shown in Figure 1(a), PAI-1, an inflammation marker, was significantly increased by HG but decreased after RSV treatment from 12 h to 48 h, accompanied by p-Akt/Akt ratio elevation from 10 min to 24 h (Figure 1(b)).

3.2. RSV Attenuated HG-Induced PAI-1 Expression and Cell Proliferation In Vitro, Which Might Be Akt/NF- κ B Pathway Dependent. To determine the relationship between increased

TABLE 1: The effects of RSV on biochemical parameters in diabetes mice.

	Control (<i>n</i> = 6)	DM (<i>n</i> = 8)	DM/RSV (<i>n</i> = 8)
Blood glucose (mg/dL)	167.59 ± 19.20	360.63 ± 86.32*	309.49 ± 76.87*
Blood urea nitrogen (mg/dL)	18.42 ± 2.62	43.81 ± 10.16*	37.69 ± 8.69*
Plasma creatinine (mg/dL)	0.22 ± 0.04	0.29 ± 0.03*	0.25 ± 0.04*
ACR (μg/mg)	38.4 ± 5.3	114.6 ± 67.2*	62.2 ± 19.6 [#]

Notes: values were expressed as means ± SD (*n* = 6~8 per group). **P* < 0.05 versus control group; [#]*P* < 0.05 versus DM group. RSV: resveratrol; DM: diabetes mellitus.

PAI-1 expression and Akt activation, the Akt activity inhibitors, LY, and MK were used. After 24 h of treatment, cells were harvested for analysis. As shown in Figure 2, PAI-1 expression (Figures 2(a) and 2(b)) and cell proliferation (Figures 2(g) and 2(h)) were significantly increased in HG group, and these changes were abolished by either RSV or Akt activity inhibitors treatment, suggesting HG-induced PAI-1 over-expression and mesangial cell proliferation through PI3K/Akt signaling pathway. This hypothesis was confirmed by further detecting of p-Akt/Akt ratio and NF-κB, a downstream target of Akt. Similar to the change of PAI-1 and cell proliferation, increased p-Akt/Akt ratio (Figures 2(c) and 2(d)) and NF-κB (Figures 2(e) and 2(f)) protein levels in HG group were also reversed by either RSV or Akt activity inhibitors.

3.3. RSV Protected Mice from Diabetes-Induced Kidney Dysfunctional and Structural Changes In Vivo. To determine the effects of RSV on the development of diabetes-induced kidney damage, STZ-induced-diabetes mouse model was utilized. After STZ injection, RSV (10 mg/Kg) was given by gavage administration once daily for 12 weeks. At the end of the experiment, mice were killed and blood, urine, and kidney tissue were harvested. RSV-treated diabetes mice developed similar levels of blood glucose, urea nitrogen, and serum creatinine as diabetes mice. However, ACR was significantly decreased in RSV-treated DM group (Table 1). Additionally, we found that mice in DM group developed renal hypertrophy with increased kidney weight to body weight ratio (Figure 3(a)) and increase glomerular area and extracellular matrix (ECM) accumulation (Figure 3(b)), while RSV treatment significantly prevented glomerular enlargement.

3.4. RSV Downregulated Akt/NF-κB Pathway in Diabetes Mouse Kidney In Vivo. As shown in Figure 4, kidney p-Akt/Akt ratio (Figure 4(a)) and NF-κB (Figure 4(b)) were significantly increased in DM group but not in RSV-treated DM group. These data were consistency with the *in vitro* results and further confirmed our hypothesis.

3.5. RSV Protected Mice from Diabetes-Induced Kidney Inflammation and Cell Proliferation In Vivo. Besides Akt/NF-κB pathway changes, PAI-1 (Figure 5(a)) and ICAM-1 (Figure 5(b)) were also increased in diabetes kidney in the protein levels. In addition, PCNA, a marker of cell proliferation, was also detected. As shown in Figure 6, kidney PCNA

mRNA (Figure 6(a)) and the number of positive cells found in glomeruli (Figure 6(b)) were significantly increased in diabetes mice compared with those in control mice. However, these changes were reversed by RSV.

4. Discussion

The present study investigated the renoprotective potential of RSV against hyperglycemia-mediated inflammation and mesangial cell proliferation both *in vitro* and *in vivo* and revealed the following innovative findings. Primarily, our data provide confirmatory evidence that RSV treatment may attenuate kidney inflammation and mesangial cell proliferation in diabetes model both *in vivo* and *in vitro*. Secondly, both RSV and Akt activity inhibitors reduced the high glucose-induced upregulation of mesangial cell proliferation as well as PAI-1 and ICAM-1 protein levels, which was Akt/NF-κB pathway dependent.

The alteration of Akt activity in DM takes part in the pathophysiology of diabetic microvascular complications. Studies focusing on Akt in diabetes suggested both decrease and increase in Akt activity in DM [13]. Several studies reported that Akt activity was increased in DN [14, 15], which was consistent with our current result and could be downregulated by RSV. Yet, to the best of our knowledge, such observation has not yet been reported in renal mesangial cells. Furthermore, active Akt is considered as one of physiological activators of NF-κB [13]. Sheu et al. reported that PI3K inhibitors effectively attenuated HG-mediated NF-κB activation in mesangial cells [16]. Consistent with above observations, we found that both RSV and Akt activity inhibitors could inhibit HG-induced NF-κB overexpression in mesangial cells. Taken together, the beneficial effect of RSV on DN might be associated with deactivation of Akt-NF-κB pathway.

Recent studies have established that RSV has protective effects on the development of DN in animals by interacting with different targets, including Akt [8–10, 17–22]. However, the mechanism by which RSV decreases Akt phosphorylation remains not fully understood. In Kim et al.'s study, the PI3K-Akt pathway suppression by RSV was due to the activation of AMPK-Sirt1-PGC-1α pathway in kidney *in vivo* [8]. Another study provided a MAPK-Sirt1-PGC-1α independent pathway. Fröjdö et al. claimed that RSV targets class IA PI3Ks by directly binding to the p110α and p110β catalytic lysine residues of PI3K and consequently inhibits their downstream signaling molecules-Akt [23]. To determine the exact

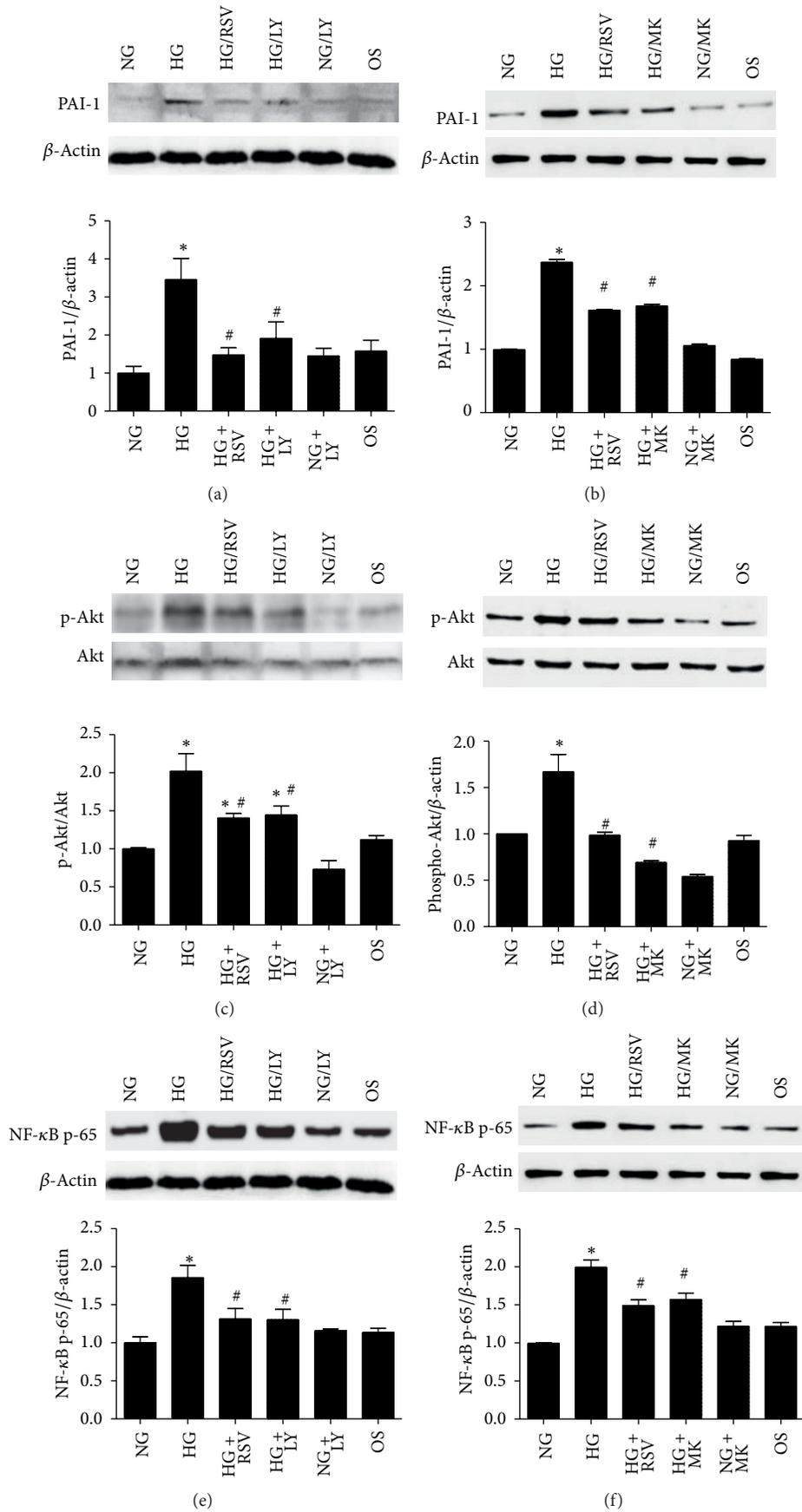


FIGURE 2: Continued.

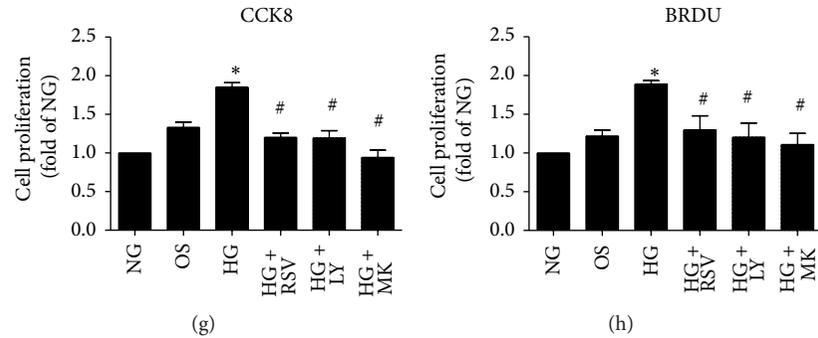


FIGURE 2: Resveratrol (RSV) attenuated high glucose- (HG-) induced plasminogen activator inhibitor (PAI-1) expression and cell proliferation in rat mesangial cell (RMC), which might be Akt/nuclear factor-kappa B (NF- κ B) pathway dependent. RMCs were cultured in DMEM containing 5.6 mM glucose (NG) and 10% FBS. When the confluence reached at 60%–70%, the medium was replaced with NG and 0.2% BSA. 24 h later, the cells were pretreated with 10 μ M LY294002, 1 μ M MK-2206, or an equal volume of DMSO for 30 min and then incubated for another 24 h with or without HG (25 mM) in the presence or absence of RSV (25 μ M). After that, cells were collected and protein levels of PAI-1 ((a), (b)), Akt, p-Akt ((c), (d)), and NF- κ B ((e), (f)) were detected using Western blotting assay. Additionally, cell proliferation was examined with CCK8 assay and BrdU incorporation, respectively. Results represent as mean \pm SD. * P < 0.05 compared with NG, # P < 0.05 compared with HG.

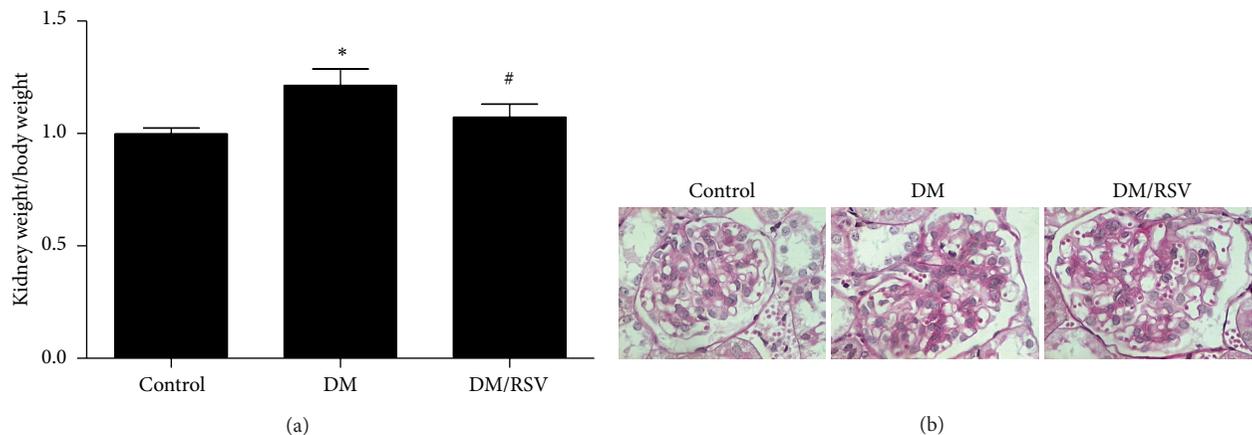


FIGURE 3: Resveratrol (RSV) protected mice from diabetes-induced renal hypertrophy and structural changes in mice. Kidney weight to body weight ratio (a) of three groups was presented. PAS staining of rat glomeruli sections ($\times 400$) was shown as Figure 3(b). Results represent as mean \pm SD. * P < 0.05 compared with control group, # P < 0.05 compared with diabetes mellitus (DM) group.

mechanism whereby RSV deactivates Akt activity, further studies are needed.

Increasing data suggest a pivotal role for NF- κ B in a variety of pathophysiological conditions in which either inflammation or cell number control is critical events. Most of the current clinical and experimental strategies to reduce the progression of DN, such as renin-angiotensin system inhibitors [24], thiazolidinedione [25], and statins [26], are known to modulate NF- κ B [27]. NF- κ B promotes the expression of a number of genes involved in inflammation, such as PAI-1 and ICAM-1 [28]. DN is characterized by excessive accumulation of ECM in the kidney. PAI-1 plays an important role in ECM remodeling through increased ECM synthesis as well as decreased ECM degradation [29]. It is also reported that PAI-1 is critically involved in inflammatory responses associated with NF- κ B pathway in kidneys from diabetic rats [3]. ICAM-1, which is induced in HG-treated renal

mesangial cell through a NF- κ B dependent way [4], promotes inflammation by enhancing leukocyte infiltration and is involved in the pathogenesis of DN [30]. Similarly deficiency of ICAM-1 resulted in a substantial decrease in macrophage accumulation in the glomeruli leading in turn to a reduction in glomerular hypertrophy and interstitial fibrosis in ICAM-1 deficient *db/db* mice [31]. In the present study, we provided evidence here that exposure to HG significantly increased the expression of NF- κ B and its downstream gene PAI-1. RSV treatment significantly inhibited elevation of these two pathogenic cytokine factors in renal mesangial cells. Furthermore, the administration of Akt activity inhibitors greatly diminished the expression of NF- κ B and PAI-1. Similarly, in diabetic mice, RSV treatment improved renal hypertrophy, accumulation of ECM, and albuminuria by downregulating the expression of NF- κ B, PAI-1, and ICAM-1.

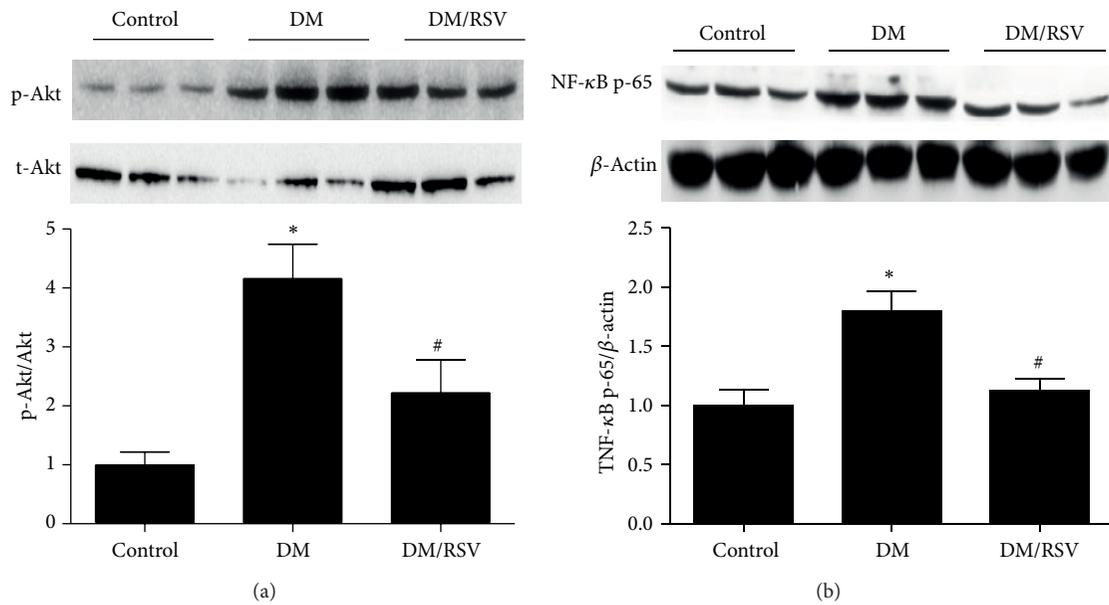


FIGURE 4: Resveratrol (RSV) downregulated Akt/nuclear factor-kappa B (NF-κB) pathway in diabetes mouse kidney. Western blot analysis was carried out to measure the protein levels of Akt, p-Akt (a) and NF-κB p65 (b). Results represent as mean ± SD. * $P < 0.05$ compared with control group, # $P < 0.05$ compared with diabetes mellitus (DM) group.

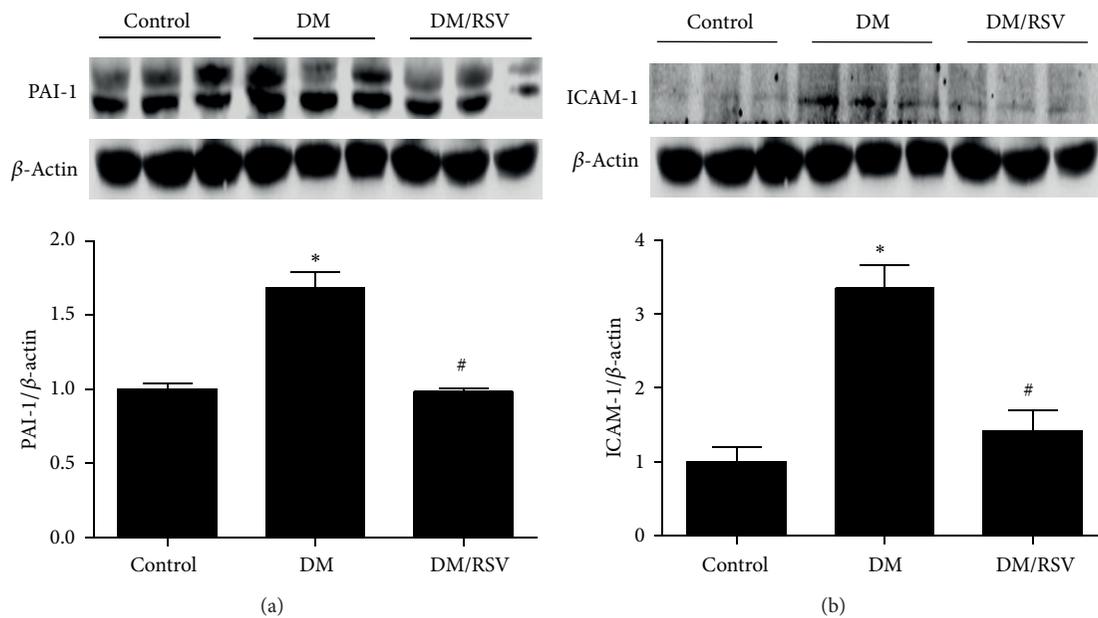


FIGURE 5: Resveratrol (RSV) protected mice from diabetes-induced kidney inflammation. Western blot analysis was carried out to measure the protein levels of plasminogen activator inhibitor (PAI-1) (a) and intercellular adhesion molecule-1 (ICAM-1) (b). Results represent as mean ± SD. * $P < 0.05$ compared with control group, # $P < 0.05$ compared with diabetes mellitus (DM) group.

In recent years, more and more data suggest that NF-κB may play an important role in the control of cell proliferation [32, 33]. It was reported that RSV could inhibit HG-induced renal mesangial cell proliferation through NF-κB pathway [10]. In our current study, we confirmed this point *in vitro*. Meanwhile, we also reported that the Akt activity inhibitors could also inhibit HG-induced renal mesangial

cell proliferation and NF-κB activity. As indicated above, the HG-induced Akt activity was downregulated by RSV and Akt activity inhibitors. Based on these, we demonstrated that RSV might be through Akt/NF-κB pathway to inhibit renal mesangial cell proliferation. Consistent with this view, in our *in vivo* study, we observed that the number of PCNA-positive mesangial cells in glomerulus and PCNA mRNA level in DM

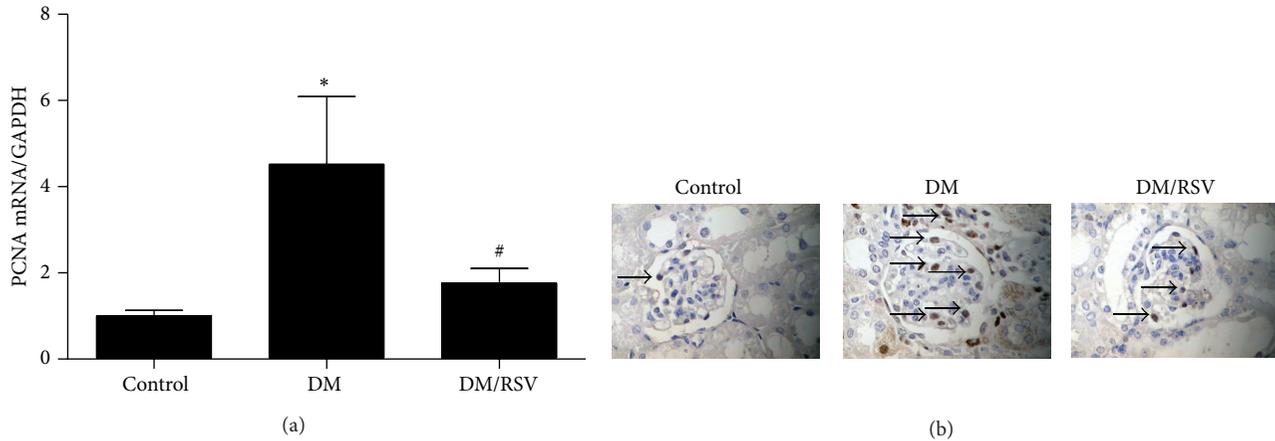


FIGURE 6: Resveratrol (RSV) protected mice from diabetes-induced mesangial cell proliferation in glomeruli. The proliferating cell nuclear antigen (PCNA) mRNA levels were detected using Real-time PCR (a). The representative images showed PCNA-positive cells (with brown nuclear) in kidney of three groups ($\times 400$) (b). Results represent as mean \pm SD. * $P < 0.05$ compared with control group, # $P < 0.05$ compared with diabetes mellitus (DM) group.

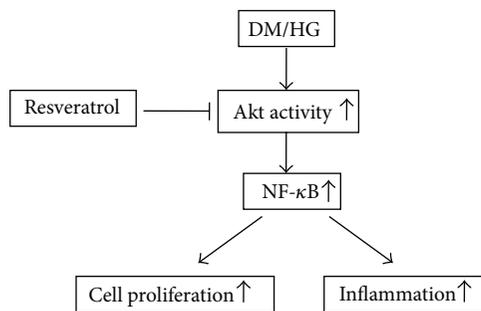


FIGURE 7: Schematic representation of proposed intracellular signaling leading to renoprotective potential of resveratrol against hyperglycemia-mediated inflammation and mesangial cell proliferation in diabetes.

group were increased compared with those in control group, which was supported by a previous study [14]. These changes could be attenuated by RSV treatment. To our knowledge, this is the first *in vivo* evidence that RSV protects DN by reducing mesangial cell proliferation.

5. Conclusion

Collectively, as indicated in Figure 7, our results showed augmented p-Akt/Akt in HG-treated mesangial cells, and such induction seemed to be attenuated by RSV and Akt activity inhibitors. Furthermore, in our *in vivo* study, we demonstrated that p-Akt increased in whole kidney lysates from 3-month STZ-induced diabetes mice and RSV treatment downregulated p-Akt expression. Since active Akt is one of physiological activators of NF-κB [13], it is possible that RSV inhibited NF-κB activity via suppression of Akt activity and, consequently, attenuated inflammation and renal mesangial cell proliferation to protect DN.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

MicroRNAs in Diabetic Kidney Disease

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Rapid growth of diabetes and diabetic kidney disease exerts a great burden on society. Owing to the lack of effective treatments for diabetic kidney disease, treatment relies on drugs that either reduces its progression or involve renal replacement therapies, such as dialysis and kidney transplantation. It is urgent to search for biomarkers for early diagnosis and effective therapy. The discovery of microRNAs had lead to a new era of post-transcriptional regulators of gene expression. Studies from cells, experimental animal models and patients under diabetic conditions demonstrate that expression patterns of microRNAs are altered during the progression of diabetic kidney disease. Functional studies indicate that the ability of microRNAs to bind 3' untranslated region of messenger RNA not only shows their capability to regulate expression of target genes, but also their therapeutic potential to diabetic kidney disease. The presence of microRNAs in plasma, serum, and urine has been shown to be possible biomarkers in diabetic kidney disease. Therefore, identification of the pathogenic role of microRNAs possesses an important clinical impact in terms of prevention and treatment of progression in diabetic kidney disease because it allows us to design novel and specific therapies and diagnostic tools for diabetic kidney disease.

1. Epidemiology and Diabetes

The prevalence of diabetes is rising worldwide and is expected to reach the devastating number of 439 million by the year 2030 from 285 million in 2010 [1]. This huge elevation is attributed to an escalating tendency towards sedentary lifestyle and westernized choice of diet, leading to obesity. Furthermore, the age of onset for the type 2 diabetic patients is showing a trend to begin in youths [2]. Diabetes is a significant public health concern as its rising incidence has greatly increased the cost of treating both diabetes and its numerous debilitating complications.

2. Diabetic Kidney Disease

Diabetic kidney disease is one of the diabetic microvascular complications. Type 1 and type 2 diabetes are distinct in etiology and pathogenesis. In spite of different morphological

changes of renal injury in type 1 and type 2 diabetic patients [3], type 1 and type 2 diabetic patients have similar risks of renal injury in diseased kidney [4]. The characteristics of diabetic renal injury includes the effacement of podocyte foot processes, gradual mesangial cell (MC) proliferation and hypertrophy, excessive accumulation of extracellular matrix (ECM) proteins, mesangial expansion, and thickening of the glomerular basement membrane (GBM) [5]. These events eventually lead to nodular glomerulosclerosis (Kimmelstiel-Wilson lesions) [6]. Similar changes occur in the tubulointerstitial, such as tubular hypertrophy, thickening of the tubular basement membrane (TBM), and interstitial fibrosis [6].

The clinical manifestations of diabetic kidney disease are microalbuminuria (30–300 mg/day), followed by macroalbuminuria (>300 mg/day), gradual loss of renal function, and elevation of arterial blood pressure and terminated in renal failure for some patients. Current interventions of diabetic kidney disease including rigorous glycemic control

and antihypertensive therapy and angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) are the first-line drugs. ACEIs and ARBs have been shown to slow down the progression of kidney disease [7–11]. However, there is no effective therapy to halt the progression to the end-stage renal disease (ESRD) after the nephropathy has been established [12].

In addition, diabetic kidney disease is also associated with macrovascular diseases, such as cardiovascular disease. In USA, diabetic kidney disease accounts for almost 50% of all ESRD [6]. Dialysis or kidney transplantation is useful to control uraemia and other symptoms of renal failure in patients with ESRD. But the prognosis for patients with ESRD due to diabetes is not optimistic because less than 50% of patients survive beyond 5 years after diagnosis [13] and the five-year survival rates are similar to those among patients with metastasized gastrointestinal carcinoma [12]. Given the ever-increasing population with diagnosed diabetes and irreversible renal injury after onset of diabetic kidney disease, to develop effective therapy is an urgent need to combat diabetic kidney diseases.

3. MicroRNAs

MicroRNAs are short noncoding RNAs 22–25 nucleotides long. As an endogenous production transcript, microRNAs can bind to the 3' untranslated region (3'UTR) of its target messenger RNA (mRNA) by imperfect complementary manner, leading to posttranscriptional gene silencing. As a result, microRNAs can inhibit gene expression via mRNA degradation, translation inhibition, or transcriptional inhibition [14, 15]. After the discovery of the first microRNA two decades ago, our knowledge of gene regulation and disease mechanisms has been renovated extensively. Nowadays, the critical role of microRNAs has been established in several cellular and biologic processes, such as proliferation, differentiation, and development, and in the regulation of genes relation to immune responses, cancer, and insulin secretion [16–19]. Because microRNAs are vital regulators of gene expression, aberrant of microRNAs are present in human diseases including cancer, hepatitis, and diabetes [17, 20–23]. There are also emerging reports about microRNAs in renal field. Several comprehensive reviews of microRNAs research on kidney development, function, and diseases have been previously published [24–39]. This review will focus on the current research progress of microRNAs in diabetic kidney disease.

4. MicroRNAs in the Pathogenesis of Diabetic Kidney Disease

The two cornerstones of progressive diabetic kidney disease are glomerulosclerosis and interstitial fibrosis. Injury of MCs and tubular epithelial cells (TECs) naturally contributed to fibrosis in diabetic kidney disease. Aberrant glucose metabolism results in accumulation of various byproducts such as advanced glycation end products (AGEs), elevation of reactive oxygen species (ROS) and activation of protein

kinase C (PKC). All of these events induce TGF- β signaling to induce renal fibrosis. Thus, hyperglycemia as a trigger initiates the cascade of renal injury in diabetic kidney disease.

In the biosynthetic process of microRNAs, Dicer is involved in both microRNAs biogenesis and microRNAs-mediated gene silencing [40–43]. As podocytes in the glomerular basement membrane are critical in maintaining the glomerular filtration barrier, podocyte dysfunction will lead to glomerular pathologies as in diabetic nephropathy (DN) or other types of glomerulonephritis [44]. Studies from mice with podocyte-specific deletion of Dicer [45–47] demonstrate that the loss of microRNAs resulted from deletion of Dicer leads to proteinuria, podocyte injury, and renal fibrosis. These results suggest the critical role of microRNAs in the podocytes to maintain the normal renal functions. Today, emerging evidences indicate microRNAs as vital regulators of gene expression during diabetic kidney disease. MicroRNAs involved in diabetic kidney disease were listed in Tables 1 and 2.

4.1. miR-192. The first landmark report about the role of microRNA in diabetic kidney disease was performed by Kato et al. [48]. They show that miR-192 levels increase significantly in glomeruli isolated from streptozotocin-injected diabetic mice as well as diabetic db/db mice, in parallel with increased TGF- β 1 and collagen 1a2 (Colla2) levels. Upregulation of renal miR-192 during diabetic kidney diseases is also found in db/db mice, type 2 diabetes rat, and whole blood samples of type 2 diabetes patients [49–52]. In addition, TGF- β 1 induces miR-192 levels in both mouse MCs and isolated glomeruli from both type 1 and type 2 mouse models of diabetes [48, 52]. Other studies also show miR-192 upregulation in MCs and TECs after treatment with high glucose, AGE, and TGF- β 1 [53–55]. These studies demonstrate that the elevation of renal miR-192 is highly correlated with diabetic condition.

Recent studies demonstrate several possible mechanisms of how miR-192 mediates renal fibrosis. Firstly, miR-192 targets the E-box repressor Smad-1 interacting protein (Sip-1, Sip-1 also called Zeb2) which binds E-box enhancer elements in the *Colla2* gene and then promotes collagen deposition in response to TGF- β 1 [48]. In addition, miR-192 induces expression of miR-216a and miR-217 which target PTEN [56]. Thus, miR-192 can activate Akt kinase to promote fibrosis as Akt activation in mouse MCs induces signatures of diabetic kidney disease, such as ECM gene expression, apoptosis inhibition, and hypertrophy [56]. Furthermore, Kato et al. also report that miR-216a can target Ybx1, an RNA binding protein and a component of P-bodies, and Ybx1 participates in TGF- β -induced collagen expression in mouse MCs [57].

The pathological role of miR-192 in diabetic kidney disease is recently confirmed by the miR-192 knockout (KO) mice [58]. Deletion of miR-192 gene in type I diabetic mice reduces albuminuria, proteinuria, renal fibrosis, and hypertrophy when compared to diabetic wild-type mice [58]. Taken together, these studies suggest that miR-192 plays a pathological role in diabetic kidney disease.

The results from animal models exhibit a pro-fibrotic role of miR-192 in diabetic kidney disease [48, 58, 59]. However,

TABLE 1: Summary of microRNAs in diabetic kidney disease (upregulation).

microRNA	<i>In vitro</i> (cell type)	<i>In vivo</i> (animal mode)	Target	References
miR-192	MCs (human, rat, and mouse)	STZ induced DN mice, DN in db/db mice	Sip-1	[48–50, 52–54]
miR-216a miR-217	Primary MCs (mouse)	STZ induced DN mice, DN in db/db mice	PTEN	[56]
miR-216a	Primary MCs (mouse)	STZ induced DN mice, DN in db/db mice	Ybx1	[57]
miR-200b/c	MCs (mouse)	STZ induced DN mice, DN in db/db mice	Zeb1/2	[52, 90]
miR-215	Primary MCs (mouse)	db/db mice	CTNNBIP1	[50]
miR-21	MCs (human and rat) PTEs (mouse)	OVE26 type 1 diabetic mice (12 weeks of age)	PTEN	[64]
	MCs, TECs (rat)	db/db mice (10 or 20 weeks of age)	Smad7	[63]
miR-29c	MCs (mouse)	db/db mice	Sprouty homolog-1	[49]
miR-377	MCs (human and mouse)	spontaneous [(NOD/Lt) mice] and STZ induced DN mice	PAK1 and MnSOD	[53]
miR-192 miR-29c miR-26b miR-200b	Mouse podocytes Mouse kidney Microvascular Endothelial cells			[53]

PTE: proximal tubule epithelial cells; TEC: tubular epithelial cells; MC: mesangial cells; STZ: streptozotocin; DN: diabetic nephropathy.

TABLE 2: Summary of microRNAs in diabetic kidney disease (downregulation).

microRNA	<i>In vitro</i> (cell type)	<i>In vivo</i> (animal mode)	Target	References
miR-192	PTE human (HK-2 cells)	Patients with established DN	Zeb2	[60, 61]
miR-215	Primary MCs and PTCs (rat)	STZ induced DN in APOE knockout mice (10 weeks of age)	Zeb2	[61]
miR-21	Primary MCs (mouse)	db/db mice (8 weeks of age)	PTEN	[67]
miR-200a	TEC (rat)	STZ induced DN in apolipoprotein E knockout mice	TGF- β 2	[88]
miR-29a	PTE human (HK-2 cells)		Col IVA1 and Col IVA2	[77]
miR-25	MCs (rat)	STZ induced DN rat	Nox4	[95]
miR-451	Primary MCs (mouse)	Early DN (db/db mice)	Ywhaz	[96]
miR-93	Renal microvascular Endothelial cell	db/db mice	VEGF-A	[94]
	Mouse podocytes			

PTE: proximal tubule epithelial cells; TEC: tubular epithelial cells; MC: mesangial cells; STZ: streptozotocin; DN: diabetic nephropathy.

the reverse is true in human nephropathy [60, 61]. In patients with established diabetic kidney disease, low expression of miR-192 is correlated with tubulointerstitial fibrosis and low estimated glomerular filtration rate (GFR) [60]. In HK-2 cells, a human proximal TEC line, TGF- β 1 (10 ng/mL for 96 h) reduces the expression of miR-192 and zinc finger E-box binding homeobox 1 (Zeb1) accompanied by a reduction of E-cadherin and upregulation of zinc finger E-box binding homeobox 2 (Zeb2), PAI-I, and vimentin [60]. Wang et al. also reported that decreased expression of miR-192 and miR-215 by TGF- β 1 (10 ng/mL for 72 h) in primary rat MCs and TEC, and in the renal cortex of apolipoprotein E knockout mice at 10 weeks of diabetes [61]. These observed differences

in murine models may be due to the different models and time points that were used [56, 61]. Reduced miR-192 and miR-215, which target Zeb2, are involved in TGF- β /CTGF-mediated changes in E-cadherin expression, demonstrating that miR-192/215 may not affect fibrosis by directly altering the expression of fibrotic markers and ECM proteins [61]. The different findings in expression of miR-192 in human and animal models of diabetic kidney disease reveal the complexity of signaling mechanism during diabetic kidney injury.

4.2. *miR-21*. miR-21 is another profibrotic microRNA because results of *in vitro* studies show that miR-21

expression is upregulated in TECs and MCs after treatment with TGF- β 1 or under diabetic condition [54, 62–65]. Elevation of miR-21 in renal cortices has been demonstrated in type 1 (OVE26) and type 2 (kk-ay and db/db) diabetic mouse models [63–66], although downregulation of miR-21 expression is reported during early DN in diabetic db/db mice [67]. Recently miR-21 expression has been found to be increased in kidney biopsies from diabetic patients compared to healthy controls [66]. In addition, the pro-fibrotic property of miR-21 is further confirmed by functional analyses as miR-21 positively regulates expression of ECM and α -SMA in TECs and MCs after treatment of TGF- β 1 or under diabetic condition [54, 62–65]. Overexpression of miR-21 in kidney cells also promotes but knockdown of miR-21 reduces renal inflammation under diabetic condition [63].

The exact mechanism of how miR-21 participates in diabetic renal injury may be related to its putative target genes and the activation of TGF- β signaling during diabetic condition [68, 69]. MiR-21 may activate the TGF- β canonical signaling by suppressing Smad7, an inhibitory Smad [63]. Furthermore, miR-21 may mediate the TGF- β noncanonical signaling by targeting Sprouty (SPRY) because SPRY is a potent inhibitor of Ras/MEK/ERK and then suppress TGF- β -dependent fibrogenic activities [70]. As phosphatase and tensin homolog (PTEN) is one of potential targets of miR-21 [71, 72], upregulation of Akt pathway may be another mechanism for miR-21 to participate in diabetic kidney injury. Suppression of PTEN by miR-21 is shown to induce phosphatidylinositide 3-kinases (PI3K) and Akt activity, and subsequently induces metalloproteinase-2 (MMP-2) expression [71]. The reciprocal regulation of PTEN levels and AKT1 substrate 1 (PRAS40), a negative regulator of Tor complex 1 (TORC1) activity by miR-21, is shown to mediate critical pathologic features of diabetic kidney disease [64]. During fibrosis, ECM turnover is controlled by both metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs) activities. The findings of TIMP3 as a potential miR-21 target, demonstrate that miR-21 may mediate several pathways to promote renal injury during diabetic kidney diseases [66]. Further studies should be done to clarify how miR-21 regulates inflammation as renal inflammation plays a vital role in diabetic kidney disease [63].

4.3. miR-29. Unlike miR-192 and miR-21, miR-29 is an anti-fibrotic microRNA. Expression of miR-29 family (miR-29a, miR-29b, and miR-29c) maintains a high level in normal kidney, lung, and heart [73] and its expression is dramatically reduced in animal models and human samples of fibrotic diseases in heart, lung, and kidney [74–76]. In addition, treatment with TGF- β 1 or under diabetic condition reduce the expression of the miR-29 family in cultured MCs, TECs, and podocytes and increases expression of ECM proteins [54, 74, 77, 78], indicating that miR-29 may play a protective role during renal injury.

Its protective role against fibrosis has been demonstrated in different disease models. In cell lines from heart, lung, and kidney with TGF- β treatment, overexpression of miR-29 suppresses but inhibition of miR-29 promotes expression of fibrotic markers [70, 74, 75, 78–81]. Gene delivery of miR-29b

either before or after established obstructive nephropathy successfully blocks progressive renal fibrosis in a mouse model of unilateral ureteral obstruction nephropathy [74], providing a strong support of anti-fibrotic properties of miR-29. Results from heart, lung, and kidneys also demonstrate that the anti-fibrotic effects of miR-29 are mediated through its ability to suppress the ECM-related gene transcription because more than 20 different ECM-related genes have been validated as direct targets of miR-29 by reporter gene assays and some of them are induced by TGF- β signaling [75, 80, 82].

The anti-fibrotic properties of miR-29 are also true in diabetic condition that suppression of miR-29a increased the risk of excess collagen deposition [77]. In addition, miR-29a in HK-2 cells negatively regulates collagen IV (Col IV) expression and directly targets the 3' UTRs of the collagen genes *Col IV a1* and *Col IV a2* [77]. On the other hand, miR-29c expression is increased in the diabetic kidneys from type 2 diabetic mouse model. Ectopic expression of miR-29c promotes the progression of diabetic kidney disease via targeting Sprouty homolog-1 and stimulation Rho kinase [49]. This difference may be due to the fact that different kidney disease models may have different expressions of miR-29 family. Thus, we speculate that each miR-29 family member may have distinct biological action on renal tissue remodeling.

4.4. miR-200. The well-established function of miR-200 family (miR-200a, miR-200b, miR-200c, miR-429, and miR-141) is to maintain epithelial differentiation and this ability is believed to be the mechanism for miR-200 family to suppress fibrosis [83–85]. These microRNAs are shown to be suppressed in cells that have undergone epithelial to mesenchymal transition (EMT) in response to TGF- β [84–87], suggesting that TGF- β regulates the expression of these microRNAs to promote EMT. It is generally believed that proximal TECs may undergo EMT to induce renal fibrosis [88]. However, this notion that EMT contributes to renal fibrosis has recently been challenged.

In renal TECs, treatment with TGF- β 1 and TGF- β 2 suppresses expression of the miR-200 family in a Smad signaling dependent manner [88, 89]. This reduction is further confirmed in a mouse model of diabetic kidney disease that reduction of renal miR-200a and miR-141 occurs in diabetic kidneys [88], suggesting that miR-200 family plays a protective role in diabetic kidney disease.

However, it is also found that amounts of miR-200b/c are elevated in glomeruli from type 1 (streptozotocin) and type 2 (db/db) diabetic mice and in mouse mesangial cells treated with TGF- β 1 *in vitro* [52]. Recently, inducing miR-200b and miR-200c and their target FOG2, an inhibitor of phosphatidylinositol 3-kinase activation, is shown to be one of the mechanisms of how TGF- β activates Akt in glomerular mesangial cells [90]. The reduction of FOG2 expression is observed in the glomeruli of diabetic mice and TGF- β -treated mouse MC. It is unexpected that increase of miR-200b/c levels is detected in diabetic mouse glomeruli and TGF- β -treated MC [90]. Transfection with miR-200b/c mimics in MC considerably reduces FOG2 expression and

increases cell hypertrophy which is confirmed by FOG2 knockdown in MC. In addition, suppression of FOG2 by miR-200b/c also activates ERKs, which is through PI3K activation [90]. These new findings suggest a new mechanism for TGF- β -induced Akt activation through FOG2 suppression by miR-200b/c, which results in glomerular mesangial hypertrophy during DN. However, the differences of miR-200 expression in diabetic kidneys are possibly due to the differences in the origin of cell lines examined, the treatments performed, and the use of different animal models between studies.

4.5. MicroRNAs in Glomerular Permeability and Podocytes. Podocytes are epithelial cells of the visceral layer of a renal glomerulus and they play a critical role in maintaining glomerular permselectivity, regulating the synthesis of ECM proteins in glomerular basement membrane (GBM) [5]. It is well accepted that loss of glomerular podocytes, accumulation of extracellular ECM in glomeruli, and hypertrophy and expansion in the glomerular mesangium are key events in the progression of DN [44], resulting in proteinuria and declining function.

Studies from two independent lines of Dicer KO mice generated for podocytes [45, 47] demonstrate that mutant mice developed proteinuria by three weeks after birth and progressed rapidly to end-stage kidney disease. Multiple abnormalities, including foot process effacement, irregular and split areas of the glomerular basement membrane, podocyte apoptosis and depletion, mesangial expansion, capillary dilation, and glomerulosclerosis, were observed in glomeruli of mutant mice [47], demonstrating that proper processing of microRNAs in podocytes is required.

Recently, specific deletion of Dicer in mouse podocyte reveals an enrichment of predicted miR-30 target genes among the upregulated genes [91]. miR-30s are shown to be expressed abundantly in glomerular podocytes in mice and TGF- β suppresses expression of *miR-30s* in podocytes [91]. As TGF- β expression and TGF- β signaling activated during diabetic condition, downregulation of *miR-30s* in podocyte may be responsible of the TGF- β -induced podocyte apoptosis.

Urinary podocyte excretion is observed in DN which is related to the reduced adhesive capacity during podocyte damage [92]. Recent studies demonstrate that miR-124 expression is related to adhesive capacity damage of podocyte [93]. During DN, renal miR-124 expression is upregulated in STZ-induced uninephrectomized diabetic rats and suppression of miR-124 ameliorates adhesive capacity of podocyte [93].

Comparative microRNA expression profile arrays identified miR-93 as one of the five microRNAs downregulated in glomeruli from db/db mice as well as in podocytes/microvascular endothelial cells exposed to a high glucose milieu [94]. The decrease in miR-93, targeting VEGF, may also be of relevance for increasing glomerular permeability of DN.

4.6. Other MicroRNAs. There are other microRNAs involved in pathogenesis of diabetic kidney disease and we list here the

major findings of these microRNAs. Further studies on these microRNAs may shed more light on their roles in diabetic kidney disease.

MiR-25 serves as an endogenous silencer for the NADPH oxidase 4 (*Nox4*) gene in mesangial cells [95]. Downregulation of miR-25 by high glucose in MCs or in rat diabetic kidney results in the relief of *Nox4* gene silencing and leads to increased *Nox4* expression and ROS production [95], suggesting that miR-25 may play a role in protecting the kidney from oxidative stress.

MiR-215 may participate in Wnt/ β -catenin signaling as miR-215 is dramatically upregulated under diabetic conditions both *in vitro* (MCs) and *in vivo* (db/db mice) [50] and miR-215 mediates TGF- β -induced MC activation and fibronectin expression via a β -catenin dependent pathway [50].

MiR-451 protects mesangial hypertrophy as renal miR-451 is downregulated during early diabetic kidney disease in db/db mice by regulating p38 mitogen-activated protein kinases (MAPK) signaling by targeting of *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta) [96].

MiR-377 may be involved in fibronectin production and oxidative stress during diabetic kidney disease. Its expression is elevated both in MCs (human and mouse) and in kidney of spontaneous (NOD/Lt) and STZ-induced type 1 diabetes mouse model [53]. MiR-377 targets p21-activated kinase-1 (PAK1) and manganese superoxide dismutase (MnSOD), which indirectly leads to upregulation of fibronectin [53].

MiR-93 is one of signature microRNAs in hyperglycemic conditions [94]. This microRNA is primarily expressed in glomeruli and TECs. However, its expression is inhibited in cultured podocyte cells, renal microvascular endothelial cells, and glomeruli of diabetic mice [94]. In culture, elevated expression of miR-93 reduces vascular endothelial growth factor (VEGF) expression and VEGF is a predicted target of miR-93 [94]. As VEGF targets collagen IV and fibronectin, the repression of miR-93 during diabetic kidney disease may contribute to the production of collagen and fibronectin.

Overall, the aberrant expression of these microRNAs in pathogenesis elicits the critical role of microRNAs in diabetic kidney disease.

5. Regulatory Mechanisms of MicroRNA during DN

The exact mechanism of how diabetic condition regulates microRNA expression during kidney diseases is still ongoing. It is believed that TGF- β signaling is responsible to promote synthesis of microRNAs during diabetic condition [39]. It has been reported that TGF- β signaling enhances the processing of primary transcripts of some microRNAs into its mature form by the Drosha complex [97]. Smad3 physically interacts with Drosha to promote the processing of pri-miR-21 into mature miR-21.

Our laboratory also demonstrates that TGF- β /Smad3 signaling mediates the transcription of miR-21, miR-192, miR-433, and the miR-29 family during renal diseases [55, 62,

74, 98, 99]. TGF- β inhibits miR-29 expression but stimulates miR-21 and miR-192 expression via the Smad3-dependent mechanism as demonstrated in MCs and TECs knocking down Smad2 or Smad3, or overexpressing Smad7, and in Smad2 or Smad3 KO mouse embryonic fibroblasts (MEF) [54, 55, 62, 74]. In addition, Smad3 physically interacts with Smad binding site (SBE) located in its promoters to regulate the expression of these microRNAs [55, 62, 74, 98]. The ability to regulate TGF- β /Smad3-mediated microRNAs via maintaining renal miR-29b but suppressing miR-192 and miR-21 is found to be one of the mechanism of how Smad7, an inhibitory Smad, protects kidneys from fibrosis [54, 55, 100]. This notion is also supported by the results from different mouse models of kidney diseases induced in mice lacking Smad3 or Smad7 or having conditional knockout (KO) for Smad2 or overexpressing renal Smad7 [54, 55, 62, 74, 98].

In addition, a recent study demonstrates that TGF- β stimulates a crosstalk circuit between p53 and miR-192 related to the pathogenesis of DN [58]. It is known that expression levels of TGF- β 1, p53, and miR-192 are increased in expanded glomeruli of diabetic mice [58, 101, 102]. Suppression of miR-192 function *in vivo* inhibits p53 expression in the renal cortex of control and streptozotocin injected diabetic mice [58]. Results from miR-192 KO type I diabetic mice confirm this positive relationship between miR-192 and renal expression of TGF- β and p53 [58]. All these results suggest that the TGF- β /Smad3 signaling plays an essential role of regulating microRNA expression during DN.

6. Therapeutic Potential of MicroRNAs in Diabetic Kidney Disease

In addition to the investigation of the role of microRNAs in DN, the recent focus has shifted to determine the therapeutic potential of the microRNAs in diabetic kidney disease. Restoring expression or inhibition of microRNAs in cells under diabetic condition has shown promising results in suppressing the expression of ECM [63, 65, 96]. Furthermore, *in vivo* delivery of microRNA mimics, inhibitors, or plasmids for overexpressing or knocking down microRNAs in rodent experimental models provides evidences that altering microRNA activity is a possible way to combat diabetic kidney disease. *In vivo* delivery of antagomiR-21 not only ameliorates creatinine clearance ratio and urine albumin creatinine ratio, but also decreases tissue inhibitor of metalloproteinase 1 (TIMP-1), Col IV and fibronectin proteins in kidney of diabetic mice [65]. Gene transfer of *miR-21* knockdown plasmids into the diabetic kidneys of db/db mice at age 10 weeks significantly ameliorates both microalbuminuria and renal fibrosis and inflammation at age 20 weeks [63]. Knockdown of miR-29c by a specific antisense oligonucleotide restores Sprouty-1 expression and ameliorates albuminuria and mesangial matrix expansion in the type 2 (db/db) diabetic mice [49]. Inhibition of miR-192 with modified antisense oligonucleotides significantly attenuates proteinuria in mice with diabetic kidney disease and suppresses oxidative stress and the renal fibrosis and hypertrophy [59]. Knockdown of miR-215 with antagomiR-215 restores

CTNNBIP expression and inhibits Wnt/ β -catenin signaling and expression of α -SMA and fibronectin in the db/db mouse kidney [50]. Overexpression of miR-451 inhibits glomerular MC proliferation *in vivo* [96]. These successful results from rodent diabetic kidney disease models demonstrate two important aspects. Firstly, altering microRNA activity in diabetic kidneys can hold the progression of diabetic kidney diseases which is not shown in current drug treatment. These promising results from experimental models demonstrate the possibility of applying microRNA therapy in the clinical practice. In addition to the conventional plasmid delivery, recent development of chemical modified oligonucleotides, that are stable in the circulation and can freely enter cells to bind to specific microRNA and silence it [30], provides possible and effective delivery methods to ensure the success of microRNA therapy in renal diseases. However, there are still some obstacles for microRNA therapy. Further attention may focus on the risk of off-target effects of microRNAs, specificity of delivery methods, and nonspecific immune response. Therefore, it is still a long way for clinic application with microRNA-based therapy against diabetic kidney disease.

7. MicroRNAs as Potential Biomarkers of Diabetic Kidney Disease

Circulating microRNAs in serum, plasma, and urine have been biomarkers of diseases because they can reflect a response to the pathophysiological stresses [103–106]. Investigation of using circulating microRNAs as biomarkers in diabetic kidney disease is ongoing because the delineation of variations of microRNA levels in the body fluids from patients with diabetic kidney disease may provide an understanding of the progression of the disease.

Recent study shows that, when compared urine microRNAs from type 1 patients with persistent and intermittent microalbuminuria, levels of 27 microRNAs are presented at significantly different levels in different stages of untreated nephropathy [107]. These correlations of microRNAs can be mapped to signaling pathways related to renal fibrosis during diabetic kidney disease [107]. A recent present study of assessment about microRNA expression in urinary exosomes from type 1 diabetic patients with and without incipient diabetic nephropathy demonstrates that miR-130a and miR-145 were enriched, while miR-155 and miR-424 reduced in urinary exosomes from patients with microalbuminuria [108]. Interestingly, the increase of urinary exosomal miR-145 levels in an animal model of early experimental diabetic nephropathy is paralleled by miR-145 overexpression within the glomeruli [108]. Treatment with high glucose in cultured MC also upregulates miR-145 levels in both MCs and MC-derived exosomes, suggesting a correlation between circulating microRNA and its renal expression during DN.

MicroRNAs found in circulation or urine appear to be upregulated or downregulated in the progression of diabetic kidney disease. The early detection of their presence in circulation or urine may assist the prediction of the disease course. In order to employ microRNA in the circulation

and urine as biomarker for determining the severity of diabetic kidney disease and checking the progress of recovery during treatment, the threshold of detection of microRNAs by various amplification methods should be increased. Establishment of a correlation of patterns of microRNAs that are released into the urine or blood by damaged kidneys and renal specific microRNA expression profiles will be useful in advancing this field by comprehensively determining their relevance in the pathogenesis of diabetic kidney disease.

8. Conclusion and Prospective

Recently, microRNAs have emerged as significant post-transcriptional regulators of gene expression in many human diseases [19, 21, 22, 29, 34]. In renal research, more evidence demonstrate that specific microRNAs alter renal physiology by changing expression patterns, mediating actions of TGF- β on renal fibrosis, affecting normal functions of MC, TEC, and podocyte, and inducing ECM deposition, podocyte dysfunction, and albuminuria during renal diseases [24, 28, 33, 34]. However, how microRNAs exactly mediate the diabetic renal injury is underexplored. In principle, one microRNA is capable of regulating multiple target genes. The more the reports of how microRNAs participate in renal injury are, the more their targets are identified. For example, more than eight direct targets of miR-21 and more than ten targets of miR-29 are found in renal research. It gives us a confusion which is the exact mechanism of how a specific microRNA is involved in diabetic renal disease. Up to date, target prediction programs can only be a guide line for the potential microRNA targets and the overlap between algorithms is only minimal. The real target needs to be validated experimentally. But the report about the relationship among the target genes of a specific microRNA is scarce and it is still an open field for future microRNA research. Hopefully, the rapid development of high-throughput validation and proteomic analysis can help us to identify real microRNA targets and determine the exact mechanism of microRNAs in renal diseases in near future.

As overexpression or knockdown of individual microRNAs in a specific cell have provided very useful data on their role in renal biology and pathobiology, generation of a single microRNA knockout in a whole-animal or tissue-specific manner should provide valuable information to confirm the critical role of microRNAs in renal physiology or pathology. For example, knockout mice of miR-21 and miR-192 have confirmed their pathological role in kidney diseases. Presence of these animal models should extend our understanding of how microRNAs work *in vivo*.

In addition to development of microRNA therapy and biomarkers, searching the polymorphism of microRNAs may be another important clinic approach to investigate the role of microRNAs in human diseases [109–111].

Finally, microRNAs act as important downstream effectors during diabetic kidney disease. The understanding of the specific role of microRNAs during diabetic kidney disease provides us not only a possible alternative to ameliorate

disease progression, but also putative biomarkers for predicting diabetic kidney disease.

Conflict of Interests

The authors declare that there is no conflict of interests between the authors and any mentioned identity in the submitted paper.

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Research Article

L-Arginine Supplementation in Type II Diabetic Rats Preserves Renal Function and Improves Insulin Sensitivity by Altering the Nitric Oxide Pathway

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Rat studies demonstrated that type II diabetes mellitus (T2DM) decreases both the production and bioavailability of nitric oxide (NO). L-arginine (LA) provides the precursor for the production of NO. We hypothesized that LA dietary supplementation will preserve NO production via endothelial nitric oxide synthase (eNOS) causing renal microvascular vasodilation and increased glomerular blood flow and thus increasing glomerular filtration rate (GFR). This would impede the formation of reactive oxygen species which contributes to cell damage and death. LA supplementation preserved GFR in the treated diabetic rats compared to untreated diabetic rats. We provide evidence that this effect may be due to increased levels of eNOS and urinary cyclic guanosine monophosphate, which leads to renal microvascular vasodilation. Plasma nitrotyrosine was decreased in the LA treated rats; however, plasma nitrite levels remained unaffected as expected. Marked improvements in glucose tolerance were also observed in the LA treated diabetic rats. These results demonstrate that LA supplementation preserves NO activity and may delay the onset of insulin resistance and renal dysfunction during hyperglycemic stress. These results suggest the importance of the NO pathway in consequent renal dysfunction and in the development of insulin resistance in diabetic rats.

1. Introduction

Diabetic nephropathy is the number one cause of end-stage renal failure [1]. The pathogenesis of diabetic nephropathy is thought to occur in two major stages. First, there is a vasodilation in the pre- and postglomerular arterioles, which leads to higher intraglomerular blood flow and pressure. This causes an early hyperfiltration in the nephrons and eventually damages the glomerular membrane and allows for proteins, glucose, and other molecules to be filtered by the glomerulus and then excreted in the urine. Secondly, the final irreversible stage is a vasoconstriction of the glomerular arterioles, which results in low blood flow and glomerular filtration rates [2].

The nitric oxide (NO) system has been shown to be altered in diabetes and in diabetic nephropathy [3]. Nitric oxide is a vasodilator and if it is deficient or its metabolism is altered, this affects renal function and insulin sensitivity [4]. The precursor for NO is L-arginine (LA). LA is an amino acid that is synthesized within the body and can also be found in various types of food. LA is converted to NO by nitric oxide synthase (NOS) [5]. Endothelial NOS (eNOS) results in NO release from the endothelium of blood vessels and causes vasodilation via cyclic guanosine monophosphate (cGMP) [6]. Inducible NOS (iNOS) is an isozyme that is present in an oxidative environment. High levels of iNOS produce larger

amounts of NO, which allows NO to react with superoxide forming peroxynitrite and thus leads to cell toxicity and/or death [7]. Therefore, higher levels of renal eNOS compared to iNOS would be beneficial in the late stages of diabetic nephropathy to maintain renal blood flow via vasodilation. A schematic of the NO biosynthetic pathway is provided in Figure 1.

While it is true in the early stages of diabetic nephropathy that there is renal vasodilation and hyperfiltration, it is in the later stages that glomerular filtration rate decreases and the continued availability of nitric oxide would maintain glomerular filtration rate and renal blood flow [8]. Our hypothesis is that nitric oxide bioavailability may be increased by L-arginine treatment in the later stages of diabetic nephropathy. It is upregulated by endothelial nitric oxide synthase rather than by the arginase enzyme.

LA deficiency causes endothelial inflammation and cardiovascular disorders, and dietary LA supplementation can reverse these disorders [9, 10]. In patients with type 2 diabetes mellitus (T2DM), LA supplementation resulted in a significant increase in NO concentration and total antioxidant status of the patient [11]. In a rat model of T2DM, the obese Zucker rat, it has been reported these rats had significantly lower renal function compared to the diabetic animals fed an antioxidant diet. This suggests that in an oxidative stress environment renal function declines and may be due to the increased production of peroxynitrite via iNOS resulting in cell death [12].

Therefore, we hypothesized that LA dietary supplementation will alter the NO biosynthetic pathway and preserve renal function in diabetic Wistar rats. To test this hypothesis, we utilized the Wistar rat model of T2DM [13] and evaluated the effects of LA dietary supplementation on four components of the NO pathway: renal eNOS and iNOS protein levels, urinary cGMP and plasma nitrotyrosine, and nitrite. We report the novel findings that LA supplementation preserves glomerular filtration rate via alterations in the NO pathway and improves insulin resistance in diabetic Wistar rats.

2. Materials and Methods

2.1. Type 2 Diabetic Rat Model and Groups. All of the animal work was conducted with approval from the Ohio University Institutional Animal Care and Use Committee.

Male Wistar rats, 27 in total, were purchased from Harlan Laboratories (Indianapolis, IN) at 6 weeks of age. After being acclimated to laboratory conditions for 4 days, they were randomly divided into three experimental groups ($n = 9$ rats/group). Group 1 served as the nondiabetic controls (Nondiabetic Control) and was fed a standard ad libitum diet (Purina Mills, Inc., St. Louis, MO, number 5012). Group 2 served as the type 2 diabetic controls (Diabetic Control) and was fed a high (61%) sucrose (HS) diet (Purina Mills Inc., St. Louis, MO, number 58R1). Group 3 (LA) was fed the same HS diet with additional 1 g/kg body weight L-arginine supplementation given twice daily via oral gavage (Sigma-Aldrich Corporation, St. Louis, MO, number A5131). Each

group was maintained on their respective diet for a total of 8 weeks.

2.2. Testing Protocol. Before being placed on the experimental diets, rat body weights were recorded and then taken weekly. Fasting blood glucose measurements were taken weekly using a One Touch Glucometer (Johnson and Johnson). At the initiation of the diet, each rat was placed in a metabolic cage with access to water solely for a 24-hour urine collection. Glomerular filtration rate (GFR) based on creatinine clearance was determined using urine and plasma creatinine assay kits (Cayman Chemicals, Ann Arbor, MI, number 500701 and number 700460) and urine output levels. GFR was calculated by urine concentration multiplied by the urine output, all divided by plasma concentration. cGMP urine levels were measured using a kit from Cayman Chemicals (Cayman Chemicals, Ann Arbor, MI, number 581021). Plasma nitrotyrosine measurements were made using a nitrotyrosine assay kit (Hycult Laboratories, Uden, the Netherlands, number HK501). These measurements were repeated at experimental times of 3, 6, and 8 weeks. A glucose tolerance test (GTT) was performed at week 6. Blood glucose readings for the GTT were taken at 0, 15, 30, 60, 90, 120, and 150 minutes after intraperitoneal injection of glucose. Plasma nitrite levels were measured using a chemical assay kit (Cayman Chemicals, number 780001) and were tested at weeks 0 and 8.

2.3. Postethanasia Analysis. At the end of the 8-week period, rats were euthanized and one kidney was removed; the renal medulla and renal cortex were separated and collected and stored in liquid nitrogen for renal eNOS and iNOS protein level analyses by Western blot.

2.4. Western Blot Analyses of eNOS and iNOS. Western blots were performed to assess the renal cortex and medulla protein levels of eNOS and iNOS. Short isoform-specific primary antibodies to eNOS (1:1000 dilution, SC-654, Santa Cruz Biotech, Santa Cruz, CA) and iNOS (iNOS-A, 1:2000, Alpha Diagnostics International, San Antonio, TX) were used. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, number 20320, Alpha Diagnostics International, San Antonio, TX) was subsequently applied. β -Actin was measured as an internal control using a monoclonal primary antibody (1:2000 dilution, A2228, Sigma-Aldrich Corporation, St. Louis, MO) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:30000 dilution, A9044, Sigma-Aldrich Corporation, St. Louis, MO). Membranes were detected with ECL immunoblotting detection reagents (GE Healthcare, Piscataway, NJ) and bands were quantified using a Chemi Doc chemiluminescent detection system and Quantity One software (Bio-Rad, Richmond CA). The results were expressed as NOS/ β -actin density ratio.

2.5. Statistical Analysis. As the main analytic framework multilevel modeling was applied to all dependent variables. Each model contained one between-subjects factor treatment

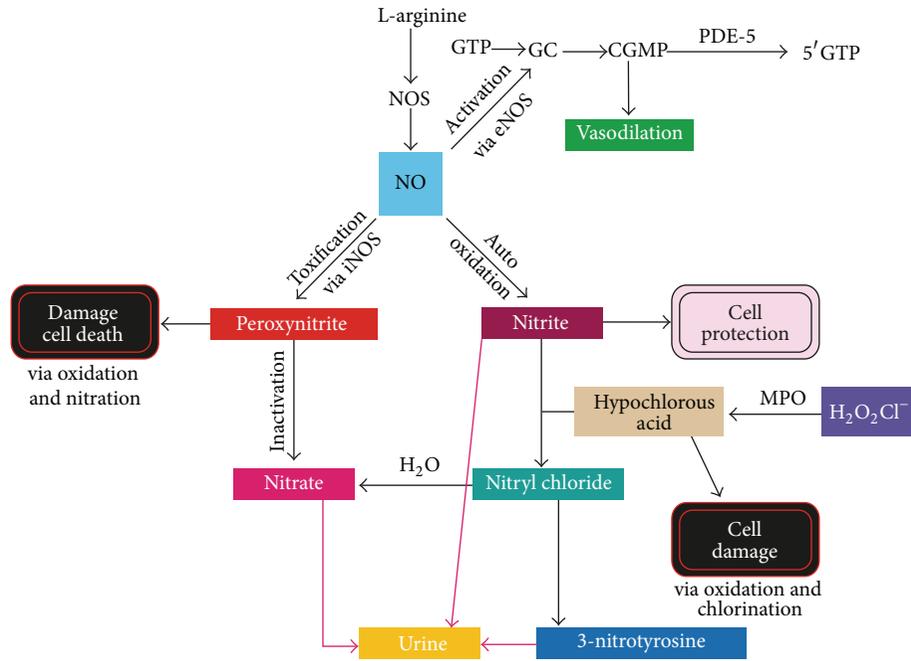


FIGURE 1: Nitric oxide biosynthetic pathway.

(TX: Control, Diabetic Control, L-arginine) and one within-subjects factor time. In each model the baseline level was included as a covariate to increase power to detect group differences. At each time point pairwise comparisons between all possible pairs of groups were performed. The significance level was set to 0.05. In the figures data are presented as means \pm standard errors.

3. Results

3.1. Weight Gain. The three groups of rats gained weight at different rates. The LA rats were heavier than the Nondiabetic Control rats at all weeks except weeks 0, 1, and 2 but were lighter than the Diabetic Control rats at all weeks (Figure 2). Similarly, the Diabetic Control rats were heavier than the Nondiabetic Control rats at all weeks except for weeks 0 and 2 (Figure 2).

3.2. Fasting Blood Glucose. Glucose levels in LA rats were higher at weeks 0 and 6 but lower at weeks 3 and 8 than those in the Nondiabetic Control rats and higher than in the Diabetic Control rats at week 0 (Figure 3). Fasting blood glucose levels in the Diabetic Control rats were higher at week 6 and lower at week 8 than those in the Nondiabetic Control rats (Figure 3). LA supplementation did not improve fasting blood glucose levels at the termination of the experimental protocol time period (Figure 3).

3.3. GFR. LA supplementation resulted in higher GFR at all time periods compared to the Nondiabetic Control rats and at weeks 3 and 8 compared to Diabetic Controls (Figure 4). GFRs in the Nondiabetic Control rats were lower than those

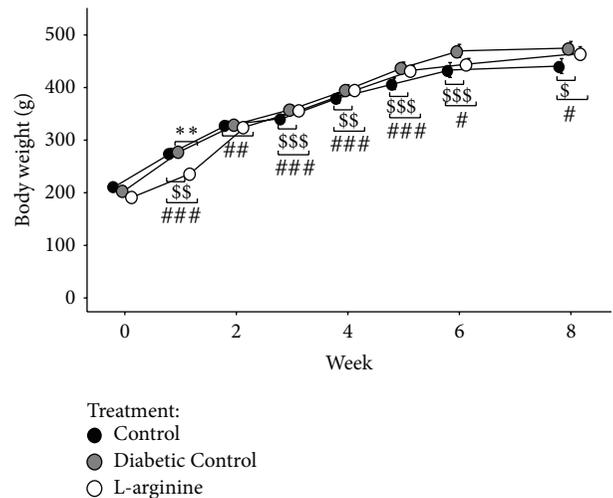


FIGURE 2: Effect of the high fructose diet alone (Diabetic Control) and high fructose plus L-arginine diet (L-Arginine) on weight gain over 8 weeks. Data are represented as means \pm standard deviations. Control versus Diabetic Control: $^{\$}P < 0.05$, $^{SS}P < 0.01$, and $^{SSS}P < 0.001$; Control versus L-arginine: $^{\#}P < 0.05$ and $^{###}P < 0.001$; Diabetic Control versus L-arginine: $^{**}P < 0.01$.

in the Diabetic Control rats at weeks 0 and 3 but were higher at weeks 6 and 8 (Figure 4).

3.4. cGMP. LA supplementation increased cGMP levels in diabetic rats. The Nondiabetic Control rats had significantly lower cGMP levels than the other 2 groups at week 6 and lower than the LA rats at week 8 (Figure 5). It is important to note that the Diabetic Control rats did not have as drastic

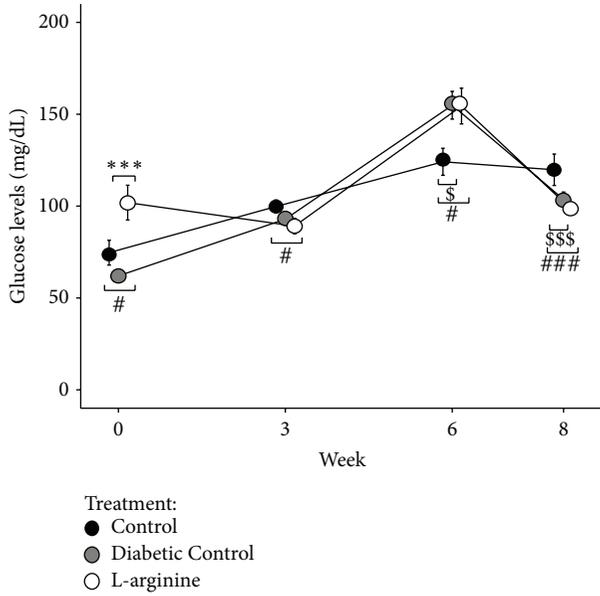


FIGURE 3: Effect of the high fructose diet alone and high fructose plus L-arginine diet on fasting glucose levels over 8 weeks. Control versus Diabetic Control: $^sP < 0.05$ and $^{sss}P < 0.001$; Control versus L-arginine: $^#P < 0.05$ and $^{###}P < 0.001$; Diabetic Control versus L-arginine: $^{***}P < 0.001$.

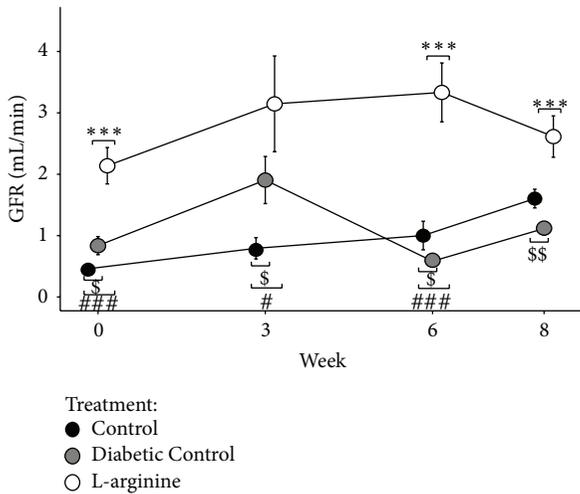


FIGURE 4: Effect of the high fructose diet alone and high fructose plus L-arginine diet on GFR over 8 weeks. Control versus Diabetic Control: $^sP < 0.05$ and $^{ss}P < 0.01$; Control versus L-arginine: $^#P < 0.05$ and $^{###}P < 0.001$; Diabetic Control versus L-arginine: $^{***}P < 0.001$.

an increase in cGMP levels as LA and Nondiabetic Control rats at weeks 6 and 8 (Figure 5).

3.5. *Relative Nitrotyrosine.* LA supplementation in diabetic rats improved plasma nitrotyrosine levels. LA rats had higher plasma nitrotyrosine levels relative to Nondiabetic Control rats at week 0, but LA rats had higher plasma nitrotyrosine levels relative to Diabetic Control rats at week 3 (Figure 6).

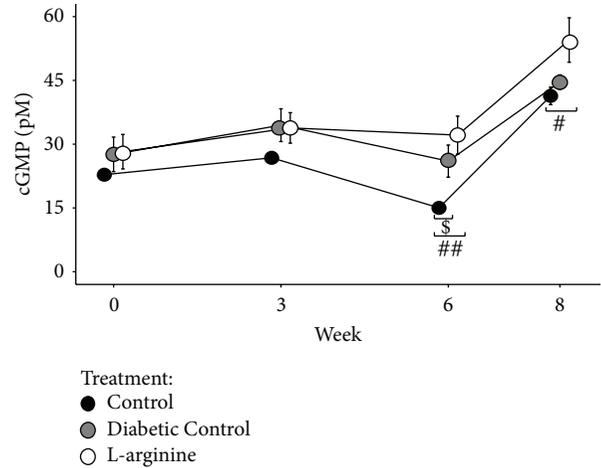


FIGURE 5: Effect of the high fructose diet alone and high fructose plus L-arginine diet on cGMP levels over 8 weeks. Control versus Diabetic Control: $^sP < 0.05$; Control versus L-arginine: $^#P < 0.05$ and $^{##}P < 0.01$.

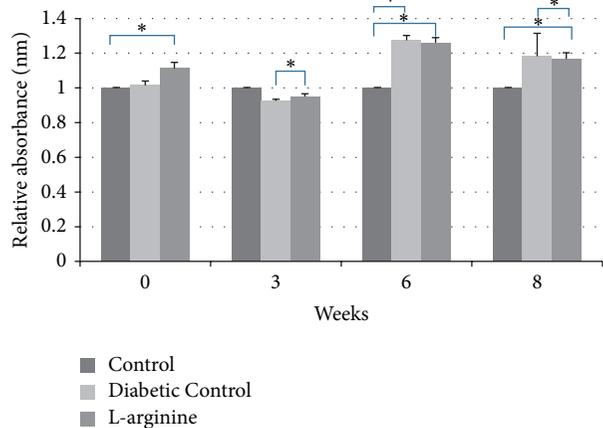


FIGURE 6: Effect of the high fructose diet alone and high fructose plus L-arginine diet on nitrotyrosine absorbance levels over 8 weeks relative to the Nondiabetic Control. Diabetic Control and L-arginine versus Control: $^*P < 0.05$.

At weeks 6 and 8, LA supplemented rats had higher plasma nitrotyrosine levels relative to Nondiabetic Control rats, but lower plasma nitrotyrosine levels relative to Diabetic Control rats (Figure 6). The Diabetic Control rats had higher plasma nitrotyrosine levels relative to both of the other groups at weeks 6 and 8 (Figure 6).

3.6. *GTT.* LA supplementation significantly improved glucose tolerance. The Diabetic Control rats had higher glucose levels than the Nondiabetic Control rats at all times after glucose challenge except for 120 minutes, while the LA rats had higher glucose levels than the Nondiabetic Control rats at 30, 60, and 90 minutes (Figure 7). Most importantly, the LA rats had lower glucose levels than the Diabetic Control rats at 15 and 30 minutes after glucose challenge (Figure 7).

TABLE 1: Effect of high sucrose diet and L-Arginine supplementation on eNOS/ β -actin ratio in kidney cortex and medulla.

N	Control		High sucrose diet		+L-arginine supplement	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla
	8	7	8	7	7	7
eNOS monomers/ β -actin	2.297 \pm 0.617	2.972 \pm 0.564	2.472 \pm 0.176	2.356 \pm 0.352	6.622 \pm 2.876	15.961 \pm 7.043 ^{1,2}
eNOS dimers/ β -actin	0.656 \pm 0.180	1.620 \pm 0.274*	0.065 \pm 0.045 ¹	0.023 \pm 0.023 ¹	0.138 \pm 0.090 ¹	0.205 \pm 0.205 ¹
eNOS total/ β -actin	2.953 \pm 0.777	4.593 \pm 0.825	2.537 \pm 0.189	2.379 \pm 0.357	6.759 \pm 2.965	16.167 \pm 7.170 ^{1,2}
Dimer/monomer ratio	0.348 \pm 0.057	0.599 \pm 0.071*	0.025 \pm 0.017 ¹	0.008 \pm 0.008 ¹	0.008 \pm 0.005 ¹	0.005 \pm 0.005 ¹

* $P < 0.05$ versus cortex, ¹ $P < 0.05$ versus control, and ² $P < 0.05$ versus high sucrose diet.

TABLE 2: Effect of a high sucrose diet and L-arginine supplementation on iNOS/ β -actin ratio in the kidney cortex and medulla.

N	Control		High sucrose diet		+L-arginine supplement	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla
	8	7	7	6	9	9
iNOS monomers/ β -actin	3.432 \pm 0.511	3.908 \pm 0.928	2.568 \pm 0.165	2.383 \pm 0.174 ¹	2.383 \pm 0.114	2.292 \pm 0.225 ¹
iNOS dimers/ β -actin	1.146 \pm 0.223	3.244 \pm 0.667*	0.105 \pm 0.071 ¹	0.419 \pm 0.262 ¹	0.011 \pm 0.007 ¹	0.042 \pm 0.040 ¹
iNOS total/ β -actin	4.578 \pm 0.654	7.151 \pm 1.540*	2.673 \pm 0.221	2.802 \pm 0.396 ¹	2.395 \pm 0.118 ¹	2.333 \pm 0.235 ¹
Dimer/monomer ratio	0.352 \pm 0.063	0.983 \pm 0.157*	0.035 \pm 0.024 ¹	0.154 \pm 0.097 ¹	0.004 \pm 0.003 ¹	0.016 \pm 0.016 ¹

* $P < 0.05$ versus cortex, ¹ $P < 0.05$ versus control, and ² $P < 0.05$ versus high sucrose diet.

for L-arginine causing renal microvascular vasodilation and increased renal blood flow and thus glomerular filtration rate. Furthermore, Morris Jr. et al. found that elevated arginase enzyme activity in the diabetic rat kidney inhibits NOS activity and NOS becomes uncoupled and this reduces the viability of NO and increases oxidative stress. This results in endothelial cell dysfunction with increased arginase enzyme activity and damage to the diabetic kidney. Therefore, we conclude that the substrate for L-arginine that maintained glomerular filtration rate in our diabetic model was eNOS and not arginase enzyme since we observed a beneficial effect and not a detrimental effect on renal function with L-arginine supplementation [18, 19].

Relative nitrotyrosine observations resulted in lower levels in the plasma for LA supplemented T2DM rats when compared to the diabetic control rats [20]. This is a positive finding in terms of the NO biosynthetic pathway because it means that there are higher levels of nitrotyrosine in the urine. Based on the NO pathway, cell damage via oxidation and chlorination would be bypassed (Figure 1). Nitrite levels remained similar between all groups, with no significant differences. This is to be expected due to auto oxidation in the NO pathway and presumably equal concentrations of nitrite that remained in the plasma and promoted cell protection. This could be further investigated by testing the plasma nitrite levels. Nitrate levels should be tested to confirm contributions from iNOS and the NO pathway. If nitrate concentrations in the urine are high, then peroxynitrite, from iNOS, has been inactivated and cell damage or death is avoided (Figure 1) [21].

There were no apparent differences among the groups in reference to body weight and fasting glucose. These findings are important because these results clearly show that alterations in the NO biosynthetic pathway precede the observance of abnormally high glucose levels and higher body

weights at the beginning of the study. However, the Diabetic Control group did increase in body weight much more rapidly than the Nondiabetic Controls as the study progressed into week 3 and beyond. The GTT shows that LA supplementation does improve glucose tolerance in diabetic rats compared to the Diabetic Controls. Blouet et al. [4] also showed improvement in glucose tolerance and insulin sensitivity in T2DM rats and reported that one of the possible mechanisms was alterations in the biosynthetic NO pathway.

Together, these findings imply that LA supplementation has beneficial effects in preserving renal function in T2DM subjects when implemented at the initial time of diagnosis. In future studies, alterations in the NO pathway need to be determined. To further prove and solidify these study's findings, the plasma and urinary nitrate, plasma cGMP, urinary nitrite, and urinary nitrotyrosine levels should be tested. Also, the different stages of diabetes and an extended experimental timeline should be considered. For example, future studies may include focusing on the effects of LA supplementation on calcium channels in the vasculature, since Awumey et al. determined that eNOS and NO production regulate extracellular calcium-induced relaxation [22]. Observing LA supplementation *in vivo* to investigate the renal microvascular vasodilatory effects to NO-mediated vasodilators would provide further insight into the mechanism of action of LA nutrient supplementation. The inexpensive dietary LA supplementation could be considered a nutritional supplement administered to T2DM patients, as an alternative to other available antidiabetic drugs.

5. Conclusions

L-arginine may be an inexpensive alternative treatment for type 2 diabetics. In this study, early intervention with L-arginine supplementation was beneficial by preserving

glomerular filtration rates, presumably via increased renal endothelial nitric oxide synthase levels leading to renal vasodilation; however, additional studies are needed to examine the alterations in the other many mediators involved in the nitric oxide pathway to further support this claim. Lastly, there was an improvement in the insulin sensitivity in the LA treated diabetic rats versus versus untreated diabetic rats.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Comparison of Two Creatinine-Based Equations for Predicting Decline in Renal Function in Type 2 Diabetic Patients with Nephropathy in a Korean Population

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Aim. To compare two creatinine-based estimated glomerular filtration rate (eGFR) equations, the chronic kidney disease epidemiology collaboration (CKD-EPI) and the modification of diet in renal disease (MDRD), for predicting the risk of CKD progression in type 2 diabetic patients with nephropathy. *Methods.* A total of 707 type 2 diabetic patients with 24 hr urinary albumin excretion of more than 30 mg/day were retrospectively recruited and traced until doubling of baseline serum creatinine (SCr) levels was noted. *Results.* During the follow-up period (median, 2.4 years), the CKD-EPI equation reclassified 10.9% of all MDRD-estimated subjects: 9.1% to an earlier stage of CKD and 1.8% to a later stage of CKD. Overall, the prevalence of CKD (eGFR < 60 mL/min/1.73 m²) was lowered from 54% to 51.6% by applying the CKD-EPI equation. On Cox-regression analysis, both equations exhibited significant associations with an increased risk for doubling of SCr. However, only the CKD-EPI equation maintained a significant hazard ratio for doubling of SCr in earlier-stage CKD (eGFR ≥ 45 mL/min/1.73 m²), when compared to stage 1 CKD (eGFR ≥ 90 mL/min/1.73 m²). *Conclusion.* In regard to CKD progression, these results suggest that the CKD-EPI equation might more accurately stratify earlier-stage CKD among type 2 diabetic patients with nephropathy than the MDRD study equation.

1. Introduction

An increasing prevalence of chronic kidney disease (CKD) is garnering greater concern worldwide [1]. Previous studies have attributed a growing trend in CKD to a rapid aging of the general population and expansion of the diabetes epidemic [1–3]. From 1991 to 2001, the incidence of diabetic nephropathy doubled among patients with newly diagnosed end stage renal disease (ESRD) [4]. Recently, an outstanding cross-sectional study including 32,208 patients with type 2 diabetes

(T2D) from 33 countries revealed that the overall prevalence of micro- and macroalbuminuria was about 39% and 10%, respectively [5]. Making matters worse, the prevalence of ESRD caused by diabetes is estimated to increase to 70% by the year 2015 [4]. T2D is well known as a leading cause of cardiovascular disease (CVD) and ESRD [6]. It is also well established that CKD has been shown to be strongly related to increased risks of CVD-related hospitalization and mortality, as well as ESRD, even after adjusting cardiovascular risk factors [1, 7, 8]. Therefore, early identification of patients

with CKD may hold additional clinical implications other than just the detection of impending progression to ESRD, especially in patients with T2D [9, 10].

Diagnosis, classification, and management of CKD are mainly dependent on overall kidney function assessed by glomerular filtration rate (GFR). To aid in the above, several creatinine-based formulas have been developed for estimating GFR. Most widely used in clinical practice, the modification of diet in renal disease (MDRD) study equation for estimating GFR was developed accounting for serum creatinine concentration, age, sex, and race [1]. Via subsequent studies, the prognostic implications of estimated GFR (eGFR) based on the MDRD study equation (eGFR MDRD) were revealed [1, 11, 12]. In accordance with these reports, eGFR MDRD has been widely known to predict the risk of ESRD in CKD patients, as well as graft failure after kidney transplant [13, 14]. Moreover, decline in eGFR MDRD has also been reported to be predictive of clinical outcomes, such as CVD events and death, particularly in patients with a CKD (GFR < 60 mL/min/1.73 m²) [1, 15, 16]. Although the MDRD study equation has generally been used for estimating GFR and evaluating CKD, imprecision and underestimation of GFR have been reported as major limitations, especially in those with early stage of CKD (GFR ≥ 60 mL/min/1.73 m²) [1, 11, 12]. In addition, in diabetic patients with microalbuminuria or overt diabetic nephropathy, it was reported that the MDRD Study equation significantly underestimated GFR [17]. Recently, the chronic kidney disease epidemiology collaboration (CKD-EPI) equation was developed utilizing a large database that pooled data from 10 studies and has been subsequently validated in 16 additional studies [18]. Based on the same four variables of the MDRD study equation, age, sex, race, and serum creatinine concentration, the CKD-EPI equation has proven to be more accurate than the MDRD Study equation in estimating GFR, especially in patients with early stage of CKD. However, there have been few studies to compare the CKD-EPI and MDRD equations with respect to the risks of clinical outcomes such as loss of kidney function or progression to ESRD in patients with T2D. Therefore, we attempted to investigate whether the CKD-EPI equation was superior to the MDRD equation in predicting decline in renal function in Korean type 2 diabetic patients with nephropathy.

2. Materials and Methods

2.1. Patients and Study Design. In this retrospective cohort study, we extracted data from an electronic medical record (EMR) database of type 2 diabetic subjects with nephropathy in whom two or more serum creatinine measurements were made between July 2000 and September 2012 at Severance Hospital in Seoul, Korea. Patients with type 2 diabetes were identified by searching the EMR database for the code ICD-10. Indicative of diabetic nephropathy, we included diabetic patients with 24 hr urinary albumin excretion ≥ 30mg/day on at least one measurement. Baseline data were defined as data measured at the point in time at which 24 hr urinary albumin excretion level exceeded 30 mg/day for the first time. Patients were excluded if they had undergone renal

replacement therapy at baseline or if they were younger than 18 years. After the baseline data extraction, patients were retrospectively followed up to two set endpoints: until May 2013 (time endpoint) or until an event of decline in renal function or death (clinical outcome endpoint). For subjects who were lost to follow-up, we included data obtained up to their final visit.

Primary outcome was evaluated according to decline in renal function and defined as doubling of baseline serum creatinine level. Doubling of baseline serum creatinine level was defined as a twofold increase in serum creatinine level for at least two consecutive measurements. This study was approved by the Institutional Review Board of Severance Hospital.

2.2. Clinical and Laboratory Measurements. Demographic and clinical findings were reviewed retrospectively for age, gender, duration of diabetes, and medications. Body mass index (BMI, kg/m²) was calculated by dividing weight (kg) by height (m) squared. Urinary albumin excretion amounts were measured with an automatic analyzer, Hitachi 7180 (Hitachi Instruments Service, Tokyo, Japan), in a 24 hr urine sample. Plasma glucose level was determined by the glucose oxidase method. HbA1c was measured by high-performance liquid chromatography using the Variant II Turbo Hemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA). Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides (TG), and creatinine measurements were performed using an autoanalyzer (Hitachi 7600; Hitachi Instruments Service, Tokyo, Japan). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula.

2.3. Estimation of GFR and Classification of CKD. The estimation of GFR was calculated using the four-variable MDRD study equation and the CKD-EPI equation [18, 19]:

$$\begin{aligned} \text{eGFR (MDRD)} &= 186.3 \times (\text{creatinine})^{-1.154} \\ &\quad \times \text{Age}^{-0.203} \times 0.742 \quad (\text{if female}), \\ \text{eGFR (CKD-EPI)} &= 141 \times \min\left(\frac{\text{creatinine}}{k}, 1\right)^\alpha \\ &\quad \times \max\left(\frac{\text{creatinine}}{k}, 1\right)^{-1.209} \\ &\quad \times 0.993^{\text{Age}} \times 1.018 \quad (\text{if female}). \end{aligned} \quad (1)$$

In the CKD-EPI equation for estimating GFR, k equals 0.7 for females and 0.9 for males; α equals -0.329 for females and -0.411 for males; min refers to the minimum value for creatinine/ k or 1; and max means the maximum for creatinine/ k or 1. For both equations, eGFR was calculated as mL/min/1.73 m², weight in kg, serum creatinine in mg/dL, and age in years. CKD stage was classified into five subgroups according to the NKF-KDOQI criteria for CKD: stage 1, eGFR ≥ 90 mL/min/1.73 m²; stage 2, eGFR of 60–89 mL/min/1.73 m²; stage 3, eGFR of 30–59 mL/min/1.73 m²; stage 4, eGFR of 15–29 mL/min/1.73 m²;

and stage 5, eGFR < 15 mL/min/1.73 m² or dialysis. Stage 3 CKD was further divided into two subgroups: stage 3a, eGFR 45–59 mL/min/1.73 m², and stage 3b, eGFR 30–44 mL/min/1.73 m² [12, 20].

2.4. Statistical Analysis. Data are presented as the means ± standard deviation. CKD was defined as an eGFR < 60 mL/min/1.73 m² for both equations for eGFR calculation [12]. Analysis of the associations between eGFR calculated by each equation and the risk of clinical outcomes was performed with Cox regression analysis after adjusting for potential confounding factors, including age, sex, diabetes duration, and HbA1c. All statistical analyses were performed with SAS version 9.2 (SAS Institute, Cary, NC, USA), and *P* values < 0.05 were considered statistically significant.

3. Results

The baseline characteristics of all 707 subjects are shown in Table 1. Mean age, HbA1c, and duration of diabetes were 61.9 ± 12.2 years, 8.2 ± 4.3 %, and 12.7 ± 8.9 years, respectively. The mean 24 hr urinary albumin excretion amount was 1094.58 ± 1867.38 mg/day, and 47.5% of the patients exhibited macroalbuminuria. The prevalence of CKD, defined as an eGFR of less than 60 mL/min/1.73 m², was 54% (*n* = 382) for the MDRD study equation and 51.6%, (*n* = 365) for the CKD-EPI equation. Oral antidiabetic drugs and insulin were used in 69.9% and 26.0% of all patients, respectively. In this study, 68.2% and 37.9% of the subjects had also taken medication for hypertension and dyslipidemia, respectively.

The most common CKD stage was stage 2 for both the MDRD study equation and the CKD-EPI equation (Table 1). Comparing CKD stage for each equation, 10.9% of MDRD-estimated patients were reclassified by adopting the CKD-EPI equation. Most reclassifications of CKD stage were observed in patients with stage 3a (eGFR MDRD of 45–59 mL/min/1.73 m²) (Figure 1). Among these patients (*n* = 107), 15.9% (*n* = 17) were reclassified to a lower stage of CKD and 0.9% were reclassified to a higher stage of CKD. Of the 229 patients with CKD stage 2 by eGFR MDRD, 13.5% (*n* = 31) were downwardly reclassified to CKD stage 1 by eGFR CKD-EPI, lowering the prevalence of CKD stage 2 from 32.4% to 31.4%. In CKD stage 3b patients with an eGFR MDRD of 30–44 mL/min/1.73 m², upward reclassification to CKD stage 3a by eGFR CKD-EPI occurred in 10.7% (*n* = 13) of 122 patients. In contrast, 7.3% (*n* = 7) of 96 patients with CKD stage 1 by eGFR MDRD were upwardly reclassified to CKD stage 2 by eGFR CKD-EPI. Overall, the prevalence of CKD (defined as an eGFR of less than 60 mL/min/1.73 m²) decreased from 54% to 51.6% by applying the CKD-EPI equation. Additionally, reclassification to an earlier stage of CKD by applying the CKD-EPI equation was likely to occur in younger subjects (median age, 55.5 versus 64.2 years, *P* < 0.001); patients reclassified to a later stage of CKD by the CKD-EPI equation were older than those who were not reclassified (median age, 76.8 versus 64.2 years, *P* < 0.001).

During a median follow-up of 2.4 years, doubling of serum creatinine level, development of ESRD, incidence of

TABLE 1: Demographic and baseline characteristics of the participants (*n* = 707).

Variables	All
Male/female	416/291
Age (years)	61.9 ± 12.2
Duration of diabetes (years)	12.7 ± 8.9
Duration of follow-up (months)	35.9 ± 49.6
HbA1c (%)	8.2 ± 4.3
Cr (mg/dL)	1.59 ± 1.24
eGFR CKD-EPI (mL/min/1.73 m ²)	59.08 ± 30.27
Stages, <i>n</i> (%)	
Stage 1: ≥90	120 (17.0)
Stage 2: 60–89	222 (31.4)
Stage 3a: 45–59	102 (14.4)
Stage 3b: 30–44	113 (16.0)
Stage 4: 15–29	104 (14.7)
Stage 5: <15	46 (6.5)
eGFR MDRD (mL/min/1.73 m ²)	58.01 ± 31.77
Stages, <i>n</i> (%)	
Stage 1: ≥90	96 (13.6)
Stage 2: 60–89	229 (32.4)
Stage 3a: 45–59	107 (15.1)
Stage 3b: 30–44	122 (17.3)
Stage 4: 15–29	111 (15.7)
Stage 5: <15	42 (5.9)
24 hr urinary albumin excretion (mg/day)	1094.58 ± 1867.38
Total cholesterol (mg/dL)	174.1 ± 53.9
Triglycerides (mg/dL)	169.6 ± 140.4
HDL cholesterol (mg/dL)	43.5 ± 15.1
LDL cholesterol (mg/dL)	99.3 ± 42.2
Oral hypoglycemic agents (%)	495 (69.9)
Insulin therapy (%)	184 (26.0)
Antihypertensive agents (%)	483 (68.2)
Lipid-lowering agents (%)	268 (37.9)

Data are shown as means ± SD or number of the case (%).

acute myocardial infarction (AMI), and stroke, as well as death from any cause, occurred in 27.9 % (*n* = 197), 13.4% (*n* = 95), 6.5% (*n* = 46), 6.5% (*n* = 46), and 10.7% (*n* = 76) of the participants, respectively. As shown in Figure 2, advance of CKD stage for both equations was associated with an increased risk for doubling of serum creatinine level in a stage-dependent manner. In Cox regression analyses (Table 2), CKD stage classified by each equation was associated with an increased risk for doubling of baseline serum creatinine levels. Comparing all CKD stages to stage 1 CKD estimated by both equations, we assessed Cox proportional hazard ratios (HRs) for doubling of serum creatinine level. For the MDRD study equation, the Cox proportional HRs for doubling of serum creatinine level were 1.54 (95% CI, 0.71–3.31; *P* = 0.27) for stage 2 and 1.79 (95% CI, 0.79–4.07; *P* = 0.17) for stage 3a. In contrast, for the CKD-EPI equation, the Cox proportional HRs for doubling of serum creatinine level were 1.90 (95% CI, 0.97–3.73; *P* = 0.063) for stage 2 and 2.18 (95% CI, 1.04–4.55; *P* = 0.038) for stage 3a. In the advanced

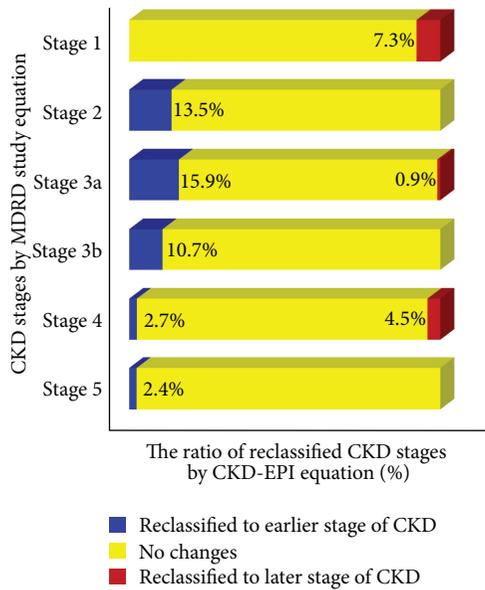


FIGURE 1: Ratio (%) of chronic kidney disease (CKD) stages for the modification of diet in renal disease (MDRD) study equation reclassified by the chronic kidney disease epidemiology collaboration (CKD-EPI) equation. Blue bars indicate reclassification to earlier stage of CKD; yellow bars, no reclassification; red bars, reclassification to later stage of CKD.

stages of CKD ($eGFR < 45 \text{ mL/min/1.73 m}^2$), both equations showed significant HRs for doubling of serum creatinine level. In model 1, the association of decreased eGFR estimated by each equation with the risk of baseline creatinine level doubling remained statistically significant after adjusting for age and sex. Additionally adjusting for duration of diabetes in model 2, duration of diabetes and HbA1c in model 3, and further adjusting for medication for hypertension in model 4, in both Cox regression models, eGFR CKD-EPI showed greater HRs for doubling of serum creatinine level than eGFR MDRD did.

4. Discussion

Considering the burden of CKD on public health worldwide, accurate estimation of renal function is of paramount importance in managing subjects with renal insufficiency, as well as in improving morbidity and mortality [18, 21]. Current clinical guidelines for CKD recommend reporting serum creatinine-based eGFR using the MDRD study formula, which includes data for age, sex, race, and serum creatinine concentration [12, 22]. Based on data from patients with CKD, the MDRD study equation is limited by imprecision and underestimation of GFR in patients of early stage of CKD ($GFR \geq 60 \text{ mL/min/1.73 m}^2$) [23, 24]. Because of these challenges, application of the MDRD study equation is considered less useful to classify patients of CKD stages 1 and 2, to verify hyperfiltration, and to track GFR changes in the higher range [1]. Furthermore, it is reported that eGFR assessed by the MDRD study equation overdiagnosed CKD,

especially in younger white women [24, 25]. A recent meta-analysis, based on various populations, revealed that not only the classification of CKD but also the risk for mortality and ESRD were more accurately predicted by the CKD-EPI equation than the MDRD study equation [26]. These unmet needs drove the advent of a new equation proposed by the CKD-EPI. Growing evidence has demonstrated that the CKD-EPI equation might be more accurate than the MDRD study equation [18, 27–29]. However, the clinical implications of eGFR assessed by the CKD-EPI equation compared to that by the MDRD study equation have not yet been well elucidated in Korean subjects with type 2 diabetes.

The present study demonstrated the superiority of the CKD-EPI equation over the MDRD study equation in identifying Korean type 2 diabetic subjects with nephropathy who were expected to show deteriorations in renal function. The present study had two main findings: first, compared with the results from eGFR MDRD, 9.1% of the type 2 diabetic subjects with nephropathy were reclassified to an earlier stage of CKD after estimation of GFR by the CKD-EPI equation. This resulted in a decrease in the prevalence of CKD stages 2, 3, and 4 from 32.4% to 31.4%, 32.4% to 30.4%, and 15.7% to 14.7%, respectively. With respect to discrepancies in CKD stage between eGFR CKD-EPI and eGFR MDRD, most studies have reported similar trends in decreased prevalence of CKD ($eGFR < 60 \text{ mL/min/1.73 m}^2$) estimated by the CKD-EPI equation in comparison to the MDRD study equation [18, 28]. Regarding precision and accuracy, one previous study demonstrated that eGFR MDRD was imprecise in patients with an $eGFR \geq 60 \text{ mL/min/1.73 m}^2$, while eGFR CKD-EPI showed less bias, improved precision, and greater accuracy than eGFR MDRD [30]. In accordance with this finding, introduction of the CKD-EPI equation in the National Health and Nutrition Examination Survey (NHANES) led to a decrease in the estimated prevalence of CKD from 13.1% to 11.5% [18]. Furthermore, in a recently conducted cohort study for subjects with T2D, the prevalence of CKD ($eGFR < 60 \text{ mL/min/1.73 m}^2$) was 22.0% for eGFR MDRD and 20.2% for eGFR CKD-EPI [31]. Because we targeted type 2 diabetic patients with nephropathy in the present study, the prevalence rate of CKD was nearly twice as high as that of the previous study. Nonetheless, similar to the previous study, we observed a decrease in the prevalence of CKD ($eGFR < 60 \text{ mL/min/1.73 m}^2$) from 54.0% to 51.6% when estimated by the CKD-EPI equation. Accordingly, we deduced that the CKD-EPI equation might allow for better risk assessment and more effective use of health care resources allocated to managing CKD-related outcomes, owing to a lower and more accurately assessed CKD prevalence [32]. Second, only the CKD-EPI equation, not the MDRD study equation, was able to predict the progression of renal insufficiency in type 2 diabetic subjects who already had albuminuria and earlier stage of CKD ($GFR \geq 45 \text{ mL/min/1.73 m}^2$). As the prevalence of earlier-stage CKD (10.8%) is more than 100 times greater than the prevalence of renal failure (0.1%), more accurate detection of CKD at earlier stages could help not only in clinical decision making but also in the allocation of public health care resources, as stated above [9]. In this regard,

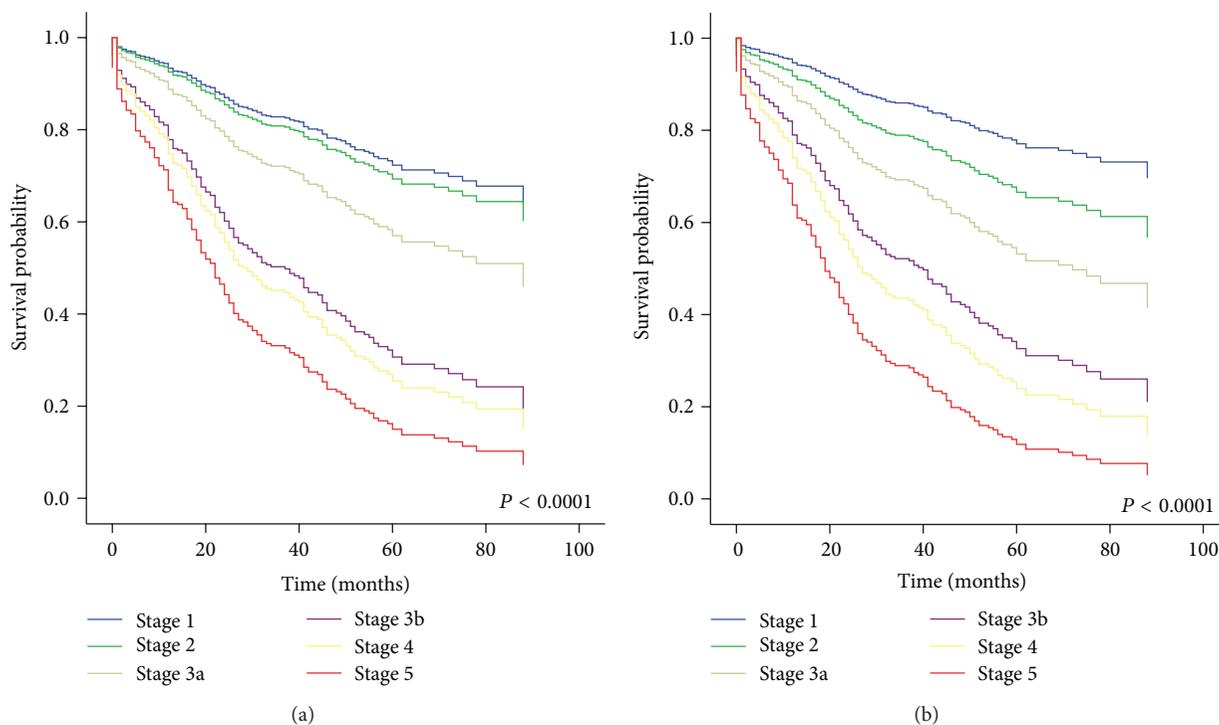


FIGURE 2: Cox-regression survival curve for doubling of serum creatinine level according to CKD stage for each eGFR equation: MDRD (a) and CKD-EPI (b). Age and sex are adjusted.

improvement in the early prediction of decline in renal function before it develops to ESRD might have significant clinical implications.

Recently, several studies in general population cohorts have demonstrated that reclassification of eGFR by the CKD-EPI equation facilitates more accurate prediction of clinical outcomes than assessment of eGFR by the MDRD study equation, in particular by shifting lower risk participants to an earlier stage of CKD [27–29]. As a practical point, the way to demonstrate the accuracy of a particular method for estimating GFR may be to evaluate its ability to predict adverse clinical outcomes [27, 32]. In other words, improvement in the ability of an eGFR equation to predict adverse outcomes may reflect more accurate estimation of GFR by said equation. In this regard, the CKD-EPI equation might be the most accurate method for estimating GFR in various populations [1, 18, 30]. Despite increasing evidence of the merits of the CKD-EPI equation, only one study to date has been performed comparing the MDRD study equation with the CKD-EPI equation for predicting adverse clinical outcomes in type 2 diabetic patients, in which the CKD-EPI equation reportedly predicted mortality more accurately than the MDRD study equation did [31]. However, in the present study, we failed to observe a significant difference in predicting all-cause mortality, ESRD, AMI, or stroke (data not shown). This might be due to the relatively small sample size and short follow-up times (median follow-up: 2.4 years). Overall, our results suggest that improved estimation of GFR by the CKD-EPI equation, compared to the MDRD study equation, allowed for better risk categorization for decline in renal function in T2D patients in Korea.

In addition to the retrospective nature of our study, there are a few important limitations that warrant consideration. First, we did not evaluate the accuracy of the two eGFR equations for estimating GFR in type 2 diabetic patients with nephropathy in comparison with directly measured GFR (e.g., GFR measurement by using inulin or isotope). Second, although the CKD-EPI equation holds greater clinical implications than the MDRD study equation in patients of an earlier stage of CKD, it still involves the inherent limitations of serum creatinine, which is dependent on muscle mass, generation, and tubular secretion [11]. Thirdly, we followed up the subjects for a relatively short term (median, 2.4 years) and had no information about potential confounding factors (e.g., smoking). Therefore, some important clinical outcomes such as ESRD or mortality could not be appropriately evaluated. Lastly, this study comprised only Korean patients with T2D, preventing our results from being generalized to other ethnic populations.

Regarding management of subjects with T2D, long-term medical complications such as CVD and ESRD should be taken into account, especially in those with diabetic nephropathy who are more prone to deteriorations in renal function and are at higher risk for comorbidities, such as CVD and mortality. In this regard, accurate prediction for possible progression to renal failure might be one of the most important clinical endpoints in evaluating diabetic patients who show the potential for unwanted clinical outcomes. In accordance with previous reports and our results, the CKD-EPI equation could be considered an optimal equation in evaluating persons with normal renal function or earlier stage of CKD, a clinical scenario similar to early

TABLE 2: Crude and adjusted Cox proportional hazard ratios for doubling of baseline serum creatinine level in 707 type 2 diabetic patients with nephropathy stratified by CKD stage according to each equation.

	HR	95% CI	P		HR	95% CI	P
CKD _{MDRD}			<0.0001	CKD _{CKD-EPI}			<0.0001
Stage 1: ≥90	1	Reference		Stage 1: ≥90	1	Reference	
Stage 2: 60–89	1.54	0.72–3.32	0.2679	Stage 2: 60–89	1.90	0.97–3.73	0.0630
Stage 3a: 45–59	1.79	0.79–4.07	0.1658	Stage 3a: 45–59	2.18	1.04–4.55	0.0383
Stage 3b: 30–44	4.11	1.92–8.82	0.0003	Stage 3b: 30–44	4.31	2.19–8.48	<0.0001
Stage 4: 15–29	4.40	2.04–9.49	0.0002	Stage 4: 15–29	5.08	2.56–10.08	<0.0001
Stage 5: <15	6.64	2.66–16.61	<0.0001	Stage 5: <15	8.09	3.65–17.95	<0.0001
Adjusted model 1			<0.0001	Adjusted model 1			<0.0001
Stage 1: ≥90	1	Reference		Stage 1: ≥90	1	Reference	
Stage 2: 60–89	1.13	0.57–2.21	0.7311	Stage 2: 60–89	1.56	0.84–2.89	0.1564
Stage 3a: 45–59	1.73	0.85–3.51	0.1324	Stage 3a: 45–59	2.42	1.26–4.67	0.0083
Stage 3b: 30–44	3.63	1.83–7.18	0.0002	Stage 3b: 30–44	4.29	2.29–8.02	<0.0001
Stage 4: 15–29	4.20	2.10–8.37	<0.0001	Stage 4: 15–29	5.47	2.89–10.35	<0.0001
Stage 5: <15	5.83	2.62–12.98	<0.0001	Stage 5: <15	8.17	3.98–16.78	<0.0001
Adjusted model 2			<0.0001	Adjusted model 2			<0.0001
Stage 1: ≥90	1	Reference		Stage 1: ≥90	1	Reference	
Stage 2: 60–89	1.12	0.55–2.28	0.7493	Stage 2: 60–89	1.70	0.88–3.28	0.1139
Stage 3a: 45–59	1.63	0.77–3.43	0.2013	Stage 3a: 45–59	2.32	1.14–4.70	0.0199
Stage 3b: 30–44	3.40	1.64–7.07	0.0010	Stage 3b: 30–44	4.39	2.22–8.67	<0.0001
Stage 4: 15–29	3.60	1.73–7.51	0.0006	Stage 4: 15–29	5.08	2.55–10.13	<0.0001
Stage 5: <15	5.34	2.27–12.55	0.0001	Stage 5: <15	7.79	3.57–17.02	<0.0001
Adjusted model 3			<0.0001	Adjusted model 3			<0.0001
Stage 1: ≥90	1	Reference		Stage 1: ≥90	1	Reference	
Stage 2: 60–89	1.09	0.54–2.23	0.8093	Stage 2: 60–89	1.67	0.86–3.23	0.1270
Stage 3a: 45–59	1.49	0.70–3.18	0.2978	Stage 3a: 45–59	2.14	1.04–4.39	0.0379
Stage 3b: 30–44	3.42	1.64–7.12	0.0010	Stage 3b: 30–44	4.43	2.24–8.76	<0.0001
Stage 4: 15–29	3.60	1.72–7.53	0.0007	Stage 4: 15–29	5.14	2.57–10.28	<0.0001
Stage 5: <15	5.39	2.26–12.88	0.0001	Stage 5: <15	8.00	3.62–17.68	<0.0001
Adjusted model 4			<0.0001	Adjusted model 4			<0.0001
Stage 1: ≥90	1	Reference		Stage 1: ≥90	1	Reference	
Stage 2: 60–89	1.25	0.56–2.81	0.5848	Stage 2: 60–89	1.90	0.91–3.97	0.0874
Stage 3a: 45–59	1.44	0.60–3.46	0.4159	Stage 3a: 45–59	2.12	0.94–4.75	0.0688
Stage 3b: 30–44	3.22	1.40–7.43	0.0060	Stage 3b: 30–44	4.03	1.88–8.63	0.0003
Stage 4: 15–29	3.22	1.43–7.71	0.0051	Stage 4: 15–29	4.77	2.19–10.37	0.0001
Stage 5: <15	6.71	2.51–17.91	0.0001	Stage 5: <15	9.82	4.05–23.80	<0.0001

CKD: chronic kidney disease; HR: hazard ratio; CI: confidence interval; MDRD: modification of diet in renal disease; CKD-EPI: chronic kidney disease epidemiology collaboration.

Model 1: adjusted for age and sex.

Model 2: adjusted for age, sex, and duration of diabetes.

Model 3: adjusted for age, sex, duration of diabetes, and baseline HbA1c level.

Model 4: adjusted for age, sex, duration of diabetes, baseline HbA1c level, and medication for hypertension.

stage diabetic nephropathy [18, 27–29]. Taken together, our findings, despite their limitations, may hold several clinical implications that warrant further investigation.

5. Conclusions

In conclusion, we suggest that the CKD-EPI equation is superior to the MDRD study equation in identifying type 2 diabetic subjects with nephropathy prone to decline in renal function. However, further studies are needed to verify the

accuracy and precision of the CKD-EPI equation compared to the MDRD study equation in estimating GFR in more diverse populations such as elderly patients, different ethnic groups, and patients with T2D [18, 33].

Disclosure

The authors alone are responsible for the content and writing of the paper.

Conflict of Interests

The authors report no conflict of interests.

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