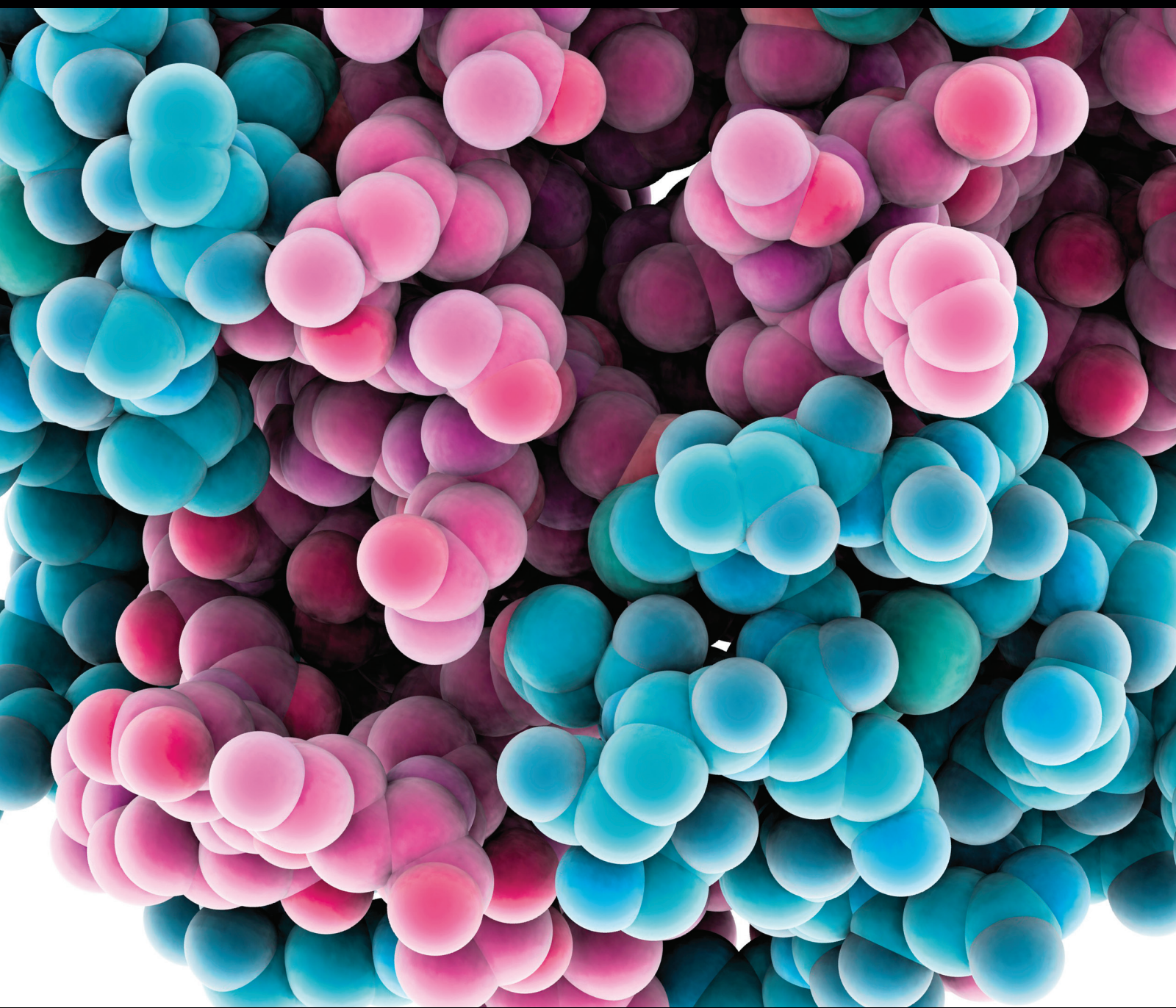
Journal of Diabetes Research

Diabetic Nephropathy: From Pathophysiology to Treatment

Lead Guest Editor: Feng Wang
Guest Editors: Yanjun Liu and Tao Xing



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Editorial Board

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Editorial

Diabetic Nephropathy: From Pathophysiology to Treatment

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Diabetic kidney disease (DKD) has been surging as the leading cause of end-stage renal disease (ESRD), as approximately one-half, in Europe, United States, Japan, and Taiwan. While in Mainland China, it has surpassed glomerulonephritis to become the number 1 cause of chronic kidney disease (CKD) in hospitalized population (1.10% versus 0.75%) in 2015 and with a substantially ascending rate doubling over the past one decade [1]. In clinical practice, some individuals with diabetes mellitus do not progress to DKD even if their blood glucose is not strictly controlled, or they are not so easily to progress from diabetic nephropathy (DN) into ESRD [2]. However, some individuals are inevitable to progress into ESRD with perfect blood glucose [3].

Thanks to international collaboration and novel analytical approaches, the underlying mechanism has been unraveled as a sophisticated model with interaction between hereditary basis and nonhereditary factors. The onset and progression of DKD are not only influenced by genetic profile but are also regulated by environmental, behavioral, and biological risk factors and their interaction with inherited predisposition. Several nonconventional risk factors may even play the critical role in the onset and progression of DKD.

The present special issue, which, including 9 original research articles and 4 review papers, has focused on the recent progress in our understanding of diabetic nephropathy including the underlying molecular mechanisms, genetic characteristics, new diagnostic biomarkers, and novel treatment options.

Studies about the genetic factors in DKD of T2DM are not so elucidated as in T1DM, since the concealing onset and the complicated phenotypes. Over the recent years, the penetration of candidate gene analysis by single-nucleotide polymorphisms (SNPs) and the more powerful genome-wide association studies (GWAS) allows dozens of genetic loci to be confirmed associated with DKD in T2DM. The efforts of screening out potential genes and SNPs are aiming to determine their role in the pathogenesis of DKD in T2DM. The localization of the gene on chromosome 18q22.3-23 was the first identified genetic loci in Turkish DKD patients of T2DM [4], which is in the region of carnosinase genes, and the polymorphism of relevant genes of carnosine dipeptidase (CNDP)1 and 2 was later proved to be related with the progression of DKD in T2DM patients [5, 6]. Two articles in this special issue targeted the genetic profiles of T2DM. Using genotyping techniques, L. Jin et al. found that the variant rs955333 was not associated with DKD, which was not consistent with the results of FIND, suggesting that the SNP might be less effective in eastern Chinese Han ancestry than other populations. In the work by T. Albrecht et al., the CNDP1 (CTG)5 homozygosity was identified as an independent, sex-specific protective factor for biopsy-proven DN. Their findings also suggested that hemodialysis patients with homozygous CNDP1 (CTG)5 genotype and diabetic patients carrying at least one (CTG)5 allele might have a survival benefit, yet to be confirmed by further studies.

Epigenetic modification also plays an important role in the pathogenesis of DN. A review by Z. Lu et al. presented recent advances in the epigenetics of DN, with the focus on the role of DNA methylation, noncoding RNAs, and histone modifications in DN [7–9].

Given that the diagnostic value of microalbuminuria in DN has recently been challenged by several studies, biomarkers other than urinary albumin are needed for the early identification of renal injury [10]. In this special issue, three clinical studies are focused on this profile. J. H. Kim et al. introduced brachial-ankle pulse wave velocity (baPWV) as a noninvasive marker associated with albuminuria in 2613 Korean patients with T2DM. A. Kamińska et al. reported that the density and size of urinary extracellular vesicles reflected renal function and could be considered as potential renal damage biomarkers in T2DM. N. Papadopoulou-Marketou et al. have conducted a small sample size cohort study to test the predictive role of serum neutrophil gelatinase-associated lipocalin (NGAL) on renal function and arterial blood pressure in T1DM. They found that serum NGAL served as an early biomarker of DN independently of microalbuminuria.

One review paper and two original research articles are related to the therapeutic options of DN. There are many ways to prevent or treat DN, but glycemic control is no doubt of paramount importance. In the clinical study titled "Renal Protective Effect of DPP-4 Inhibitors in Type 2 Diabetes Mellitus Patients: A Cohort Study," Y-G. Kim et al. studied the efficacy of dipeptidyl-peptidase IV inhibitors (DPP-4i) in preventing DN among 414 T2DM patients and found that long-term treatment of DPP-4i could delay the progression of DN by reducing urine albumin excretion and alleviating eGFR decline. Animal models are competent tools to study novel therapeutic methods including DN. In the basic research work by Y. He et al., the authors established a rat model of islet transplantation under the kidney capsule and found that successful islet transplantation protected against DN better than insulin treatment, presenting a cheerful prospect for the treatment or prevention of early DN. The review titled "Mechanistic Insight and Management of Diabetic Nephropathy: Recent Progress and Future Perspective," submitted by R. Xue et al., described recent advances about the pathophysiological process of DN and summarized emerging evidences for the management of DN.

Above all, the onset and progress of DKD is the consequence of genetic variants, epigenetic effects, and environment-involved interactions. Studies elucidating the underlying mechanisms behind DKD so far are rather limited, taking into account the paucity of well-phenotyped prospective DKD cohorts, the variable diagnostic criteria, the mosaic of ethnicity, and the cost-effective ratio of whole-genome genotyping. However, dozens of novel analysis technologies and pharmacological measures are booming out. To standardize the phenotypes, to larger the sample, to apply more stringent quality control, to replicate in multiple cohorts and to take covariates into account will no longer be a barrier in the near future. With the robust researches on the mechanisms within the onset and progression of DKD, the potential answers will ultimately be explored and will soundly shed light on the complex pathogenesis, facilitate prevention, and benefit early diagnosis and tailored intervention to reduce the incidence and minimize progression, so as to relieve the huge burden DKD posed on public health.

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Yi Fang
Feng Wang
Tao Xing

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Research Article

Arterial Stiffness Is More Associated with Albuminuria than Decreased Glomerular Filtration Rate in Patients with Type 2 Diabetes Mellitus: The REBOUND Study

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Aim. The aim of this study was to evaluate the association between arterial stiffness and albuminuria and glomerular filtration rate (GFR) in patients with type 2 diabetes mellitus. *Methods*. This multicenter cohort study analyzed 2613 patients with type 2 diabetes. Brachial-ankle pulse wave velocity (baPWV) was used as a noninvasive marker of arterial stiffness. Additionally, the patients were categorized into four groups according to their albumin-to-creatinine ratio (ACR, normoalbuminuria versus albuminuria) and estimated GFR (eGFR, <60 mL/min/1.73 m^2 versus ≥60 mL/min/1.73 m^2). *Results*. A univariate analysis revealed that maximal baPWV was significantly associated with both the ACR ($r = 0.297$, $P < 0.001$) and eGFR ($r = -0.220$, $P < 0.001$). A multivariate analysis adjusted for significant clinical variables and eGFR showed that baPWV remained significantly correlated with the ACR ($r = 0.150$, $P < 0.001$). Also, baPWV was correlated positively with the ACR in patients with an eGFR ≥ 60 mL/min/1.73 m^2 ($r = 0.146$, $P < 0.001$). However, baPWV was not correlated with eGFR after adjustment for significant clinical variables. *Conclusions*. The present findings indicate that arterial stiffness is more associated with albuminuria than a decrease in GFR in patients with type 2 diabetes mellitus.

1. Introduction

Diabetic nephropathy is one of the most serious microvascular complications that influence the mortality of diabetic patients [1]. It is estimated that 20–40% of diabetic patients are affected by this disorder, which manifests clinically as albuminuria or as a reduced glomerular filtration rate (GFR) [2–4]. Varying degrees of increased arterial stiffness are associated with different stages of chronic kidney disease (CKD) [5] and are also an independent risk factor for cardiovascular disease (CVD) and mortality [6]. Most studies have shown that arterial stiffness is independently associated with the two main components of CKD, albuminuria and a reduced GFR [7–10]. Although some studies have found that these associations can be identified in patients with type 2 diabetes [11–14], there are conflicting data regarding these relationships [15].

One previous study reported that up to 25% of patients with either type 1 or type 2 diabetes exhibit reduced renal function under conditions of a normal albumin excretion

rate (AER; <20 μg/min) [16], and subsequent studies have supported the dissociation between a decreased GFR and increased albuminuria in patients with type 2 diabetes [17–19]. Thus, the traditional paradigm of diabetic kidney disease has been challenged, and changes in the AER and GFR are being accepted as interdependent rather than essential manifestations of diabetic CKD. The discordance between changes in the AER and GFR has brought about a search for new markers that can accurately identify diabetic patients at risk of a declining GFR independent of gradual increases in AER.

The relationship between CVD and brachial-ankle pulse wave velocity (baPWV) in Korean patients with type 2 diabetes was previously investigated to assess the prognostic efficacy of baPWV for cardiovascular morbidity and mortality [20]. The primary aims of the subanalysis of that study conducted here were to determine whether arterial stiffness is associated with diabetic nephropathy and to evaluate the associations of baPWV with albuminuria and GFR as key factors underlying the development and progression of type 2 diabetic nephropathy. Additionally, whether or not baPWV has different associations with albuminuria and GFR was evaluated.

2. Methods

The REBOUND study was designed as a multicenter prospective observational study for the assessment of the association between baPWV and CVD in patients with type 2 diabetes [20]. Briefly, the REBOUND study was conducted from December 2008 to December 2010 at eight general hospitals in Busan, Korea. That study consecutively recruited 3058 patients with type 2 diabetes 30 years of age and older and measured their baPWV values, as a noninvasive marker of arterial stiffness, based on the procedures of the outpatient endocrinology departments of each hospital. The exclusion criteria were as follows: a low ankle-brachial index (ABI; <0.9), severe symptoms and/or signs of CVD, a history of acute myocardial infarction, stroke, or hospitalization for heart failure within 3 months, and chronic renal disease (serum creatinine levels > 2.0 mg/dL).

2.1. Data Collection. All data were collected from the medical records and physical examinations of the patients. Laboratory data obtained within the 3 months prior to enrollment were collected from available sources, and blood samples intended for biochemical analyses were collected after the participants fasted for at least 8 hours. Using an automatic waveform analyzer (VP-2000, Colin, Komaki, Japan), baPWV was measured automatically by the brachial-ankle distance ($L = 0.5934 \times \text{height [cm]} + 14.4014$) divided by the pulse wave time interval between the brachial region and ankle (ΔT) while the participants were kept supine for 5 minutes [21]. The right and left baPWV values were obtained, and the largest value was determined as the maximum baPWV (M-baPWV). The estimated GFR (eGFR) levels were determined using the modification of diet in renal disease (MDRD) equation: $\text{MDRD} = 186 \times (\text{serum creatinine [mg/dL]})^{-1.154} \times (\text{age in years})^{-0.203}$ [22]; an adjustment factor of 0.742 was used for women. Albuminuria (microalbuminuria and overt albuminuria) was defined based on the albumin-to-creatinine ratio (ACR; ≥30 mg/g creatinine) using random spot urine testing. The prevalence of retinopathy, neuropathy, and CVD were investigated based on medical history. Diabetic retinopathy was detected during an eye examination that includes fundus photography or ophthalmoscopy. Diabetic neuropathy was diagnosed based on symptoms, medical history, and a physical examination. CVD includes coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism.

2.2. Population and Statistical Analysis. Of the 3058 patients enrolled in the REBOUND study, 445 were excluded from the analysis due to a violation of the inclusion and/or exclusion criteria or because there were no available data for baPWV, eGFR, or ACR. In the present subanalysis, the data of 2613 patients were analyzed. The patients were categorized into four groups according to the ACR (normoalbuminuria versus albuminuria) and eGFR (<60 mL/min/1.73 m^2 versus ≥60 mL/min/1.73 m^2).

The statistical package SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was used for all data analyses. The data are presented as means ± standard deviations (SD) for normally distributed variables and as medians (interquartile ranges) for nonparametric variables. The distribution of the continuous variables was examined for skewness and kurtosis, and the logarithm-transformed values were used for the analysis. Differences among groups were analyzed by analysis of variance (ANOVA) followed by a Bonferroni's test for parametric values and the Kruskal-Wallis test for nonparametric values. Pearson's chi-squared (χ^2) test was applied to analyze categorical variables. Pearson's correlation coefficient test was used to assess the relationship between two variables. Multivariate regression analyses using either ACR or eGFR as the dependent variable and baPWV as the independent variable were conducted, and several models were used to adjust for confounding variables. A two-tailed P value < 0.05 was considered to indicate statistical significance for all statistical tests.

2.3. Ethics Statement. The protocol for the present study was approved by the institutional review boards of each hospital, including that of Pusan National University Hospital (numbers 2009041 and 20132131), and informed consent was obtained from all patients for which identifying information is included in this article.

3. Results

3.1. Patient Demographics. The demographic characteristics of the patients are shown in Table 1. The mean age of the entire population was 59.6 ± 10.7 years (range: 30–89 years), 43.4% of the population was male, 56.6% of the population was female, and the mean duration of diabetes was 9.1 ± 6.9 years. The average body mass index (BMI) was 24.9 ± 3.4 kg/m^2, the average waist circumference was 88.8

Table 1: Comparison of clinical characteristics according to albuminuria and eGFR group.

Characteristics	Normoalbuminuria		P value	Albuminuria		P value
	eGFR ≥ 60 (n = 1575)	eGFR < 60 (n = 223)		eGFR ≥ 60 (n = 559)	eGFR < 60 (n = 256)	
Age, years	58.0 ± 10.1	67.2 ± 9.2	<0.001	58.6 ± 11.3	65.1 ± 10.0	<0.001
Sex, male/female	685/890	58/165	<0.001	286/273	104/152	0.005
BMI, kg/m^2	24.7 ± 3.2	25.2 ± 3.0	0.016	25.4 ± 3.9	25.0 ± 3.4	0.318
Waist circumference, cm	88 ± 8	91 ± 9	<0.001	91 ± 10	90 ± 9	0.729
Duration of diabetes, years	7.9 ± 7.0	10.1 ± 6.8	<0.001	10.0 ± 7.7	13.3 ± 7.8	<0.001
SBP, mmHg	126 ± 14	131 ± 18	0.005	132 ± 17	140 ± 22	<0.001
DBP, mmHg	78 ± 9	76 ± 10	0.555	80 ± 10	78 ± 12	0.614
Pulse pressure, mmHg	48 ± 11	55 ± 14	<0.001	52 ± 13	61 ± 16	<0.001
Heart rate, bpm	74 ± 11	75 ± 12	0.568	78 ± 12	76 ± 12	0.093
HbA1c, %	7.4 ± 1.5	7.5 ± 1.5	0.012	8.1 ± 1.8	7.8 ± 1.8	0.771
HbA1c, mmol/mol	57.6 ± 16.8	58.4 ± 16.8	0.012	65.4 ± 19.5	62.2 ± 19.7	0.771
eGFR, mL/min/1.73 m^2	87.3 ± 21.5	52.1 ± 8.2	<0.001	85.6 ± 21.9	45.1 ± 12	<0.001
ACR, mg/g*	6.2 (3.3–12.1)	7.4 (4.3–14.2)	0.033	82.1 (44.8–193.9)	220.9 (74.7–827.6)	<0.001
LDL cholesterol, mg/dL	93 ± 33	91 ± 32	0.564	95 ± 31	92.9 ± 36	0.824
HDL cholesterol, mg/dL	49 ± 12	47 ± 12	0.001	48 ± 13	44 ± 12	<0.001
Triglyceride, mg/dL*	115 (82–164)	116 (89–166)	0.007	130 (93–200)	133 (98–182)	0.140
hsCRP, mg/dL*	0.11 (0.05–0.45)	0.19 (0.06–1.00)	<0.001	0.19 (0.08–0.91)	0.32 (0.09–1.23)	0.017
M-baPWV, cm/sec*	1531 (1363–1725)	1708 (1490–2006)	0.002	1677 (1443–1934)	1878 (1615–2161)	0.005
Right ABI	1.11 ± 0.09	1.10 ± 0.10	0.094	1.09 ± 0.12	1.08 ± 0.13	0.574
Left ABI	1.11 ± 0.09	1.11 ± 0.11	0.786	1.10 ± 0.10	1.09 ± 0.13	0.587
Smoking, %	21.6	8.7	<0.001	26.8	15.9	0.001
Alcohol consumption, %	28.5	16.8	<0.001	33.3	19.1	<0.001
Insulin treatment, %	19.5	28.3	0.002	35.1	52.3	<0.001
RAS inhibitors, %	41.6	52.5	0.001	62.1	71.1	0.012
Lipid lowering agent, %	58.5	65.9	0.036	64.2	61.3	0.426
Antiplatelet agent, %	60.6	66.4	0.096	61.5	64.1	0.490

Values are presented as mean ± SD for parametric variables and median (interquartile range) for nonparametric variables. t-test for age; chi-square test for categorical variables; age and sex-adjusted ANCOVA for all other continuous variables; *logarithm-transformed values were used for comparison. M-baPWV: maximum brachial-ankle pulse wave velocity; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; ABI: ankle-brachial index; LDL: low-density lipoprotein; HDL: high-density lipoprotein; HbA1c: hemoglobin A1c; hsCRP: high-sensitivity C-reactive protein; eGFR: estimated glomerular filtration rate.

± 8.7 cm, the average glycated hemoglobin (HbA1c) level was 7.6 ± 1.6% (59.8 ± 18.0 mmol/mol), and the prevalence rates of albuminuria and CKD of stage 3 or greater were 31.2% and 18.3%, respectively. The patients were categorized into four groups according to albuminuria status and eGFR: those with an eGFR ≥ 60 mL/min/1.73 m^2 and normoalbuminuria (n = 1575), those with an eGFR < 60 mL/min/1.73 m^2 and normoalbuminuria (n = 223), those with an eGFR ≥ 60 mL/min/1.73 m^2 and albuminuria (ACR ≥ 30 mg/g creatinine; n = 559), and those with an eGFR < 60 mL/min/1.73 m^2 and albuminuria (n = 256).

Among the normoalbuminuric patients, all variables except for diastolic blood pressure (DBP), heart rate, low-density lipoprotein (LDL) cholesterol level, and the ABI significantly differed between the two groups according to the eGFR (Table 1). The M-baPWV was higher in patients with an eGFR < 60 mL/min/1.73 m^2 than in patients with an eGFR ≥ 60 mL/min/1.73 m^2. Among the albuminuric patients, there were no differences in BMI, waist circumference, DBP, heart rate, HbA1c, LDL cholesterol, triglyceride, and ABI levels according to eGFR between the two groups. However, the M-baPWV was higher in patients with an eGFR < 60 mL/min/1.73 m^2 than in patients with an eGFR ≥ 60 mL/min/1.73 m^2. Among both normoalbuminuric and albuminuric patients, CVD was more prevalent in patients with an eGFR < 60 mL/min/1.73 m^2 than in patients with an eGFR ≥ 60 mL/min/1.73 m^2 (Table 2). Additionally, two major complications, neuropathy and retinopathy, were more frequently observed in patients with an eGFR < 60 mL/min/1.73 m^2 than in patients with an eGFR ≥ 60 mL/min/1.73 m^2.

3.2. Associations of baPWV with the ACR and eGFR. A univariate regression analysis of the entire population revealed that baPWV was significantly associated with the ACR (model 1; Table 3) and eGFR (model 1; Table 4). The patients were divided into four groups according to quartiles of M-baPWV levels, and comparison of clinical characteristics was shown in Supplementary Table 1 available online at https://doi.org/10.1155/2017/7047909. After adjusting for

Table 2: The prevalence of chronic complications according to the albuminuria and eGFR group.

Characteristics	Normoalbuminuria		P value	Albuminuria		P value
	eGFR ≥ 60 (n = 1575)	eGFR < 60 (n = 223)		eGFR ≥ 60 (n = 559)	eGFR < 60 (n = 256)	
Cardiovascular disease	7.7	18.6	<0.001	6.5	14.9	<0.001
Coronary artery disease	5.8	16.2	<0.001	4.4	11.8	<0.001
Cerebrovascular disease	2.0	4.4	0.029	2.2	2.6	0.711
Peripheral artery disease	0.8	1.5	0.315	0.4	1.3	0.201
Neuropathy	38.7	56.3	<0.001	52.2	65.9	<0.001
Retinopathy	13.4	21.4	0.003	28.9	48.6	<0.001

eGFR: estimated glomerular filtration rate.

Table 3: Multivariate regression analyses with ACR as a dependent variable and baPWV as an independent variable.

Model	All (n = 2613)		eGFR ≥ 60 (n = 2134)		eGFR < 60 (n = 479)	
	Standard β	P value	Standard β	P value	Standard β	P value
1	0.297	<0.001	0.251	<0.001	0.216	<0.001
2	0.347	<0.001	0.298	<0.001	0.364	<0.001
3	0.152	<0.001	0.145	<0.001	0.131	0.013
4	0.150	<0.001	0.146	<0.001	0.091	0.071

Model 1: crude; model 2: adjusted for age and sex; model 3: adjusted for significant clinical parameters including BMI, duration of diabetes, SBP, pulse pressure, heart rate, smoking, alcohol consumption, HbA1c, HDL cholesterol, hsCRP, insulin treatment, and RAS inhibitors; model 4: adjusted for eGFR; ACR: albumin-to-creatinine ratio; baPWV: brachial-ankle pulse wave velocity; eGFR: estimated glomerular filtration rate.

Table 4: Multivariate regression analyses with eGFR as a dependent variable and baPWV as an independent variable.

Model	All (n = 2613)		Normoalbuminuria (n = 1798)		Albuminuria (n = 815)	
	Standard β	P value	Standard β	P value	Standard β	P value
1	−0.220	<0.001	−0.141	<0.001	−0.245	<0.001
2	−0.071	0.001	0.025	0.357	−0.090	0.017
3	−0.013	0.620	0.011	0.736	0.013	0.769
4	−0.013	0.635	0.012	0.711	0.050	0.243

Model 1: crude; model 2: adjusted for age and sex; model 3: adjusted for significant clinical parameters including BMI, duration of diabetes, SBP, pulse pressure, heart rate, smoking, alcohol consumption, HbA1c, HDL cholesterol, hsCRP, insulin treatment, and RAS inhibitors; model 4: adjusted for ACR; ACR: albumin-to-creatinine ratio; baPWV: brachial-ankle pulse wave velocity; eGFR: estimated glomerular filtration rate.

age, sex, and significant clinical variables such as BMI, duration of diabetes, SBP, pulse pressure, heart rate, smoking, alcohol consumption, HbA1c, HDL cholesterol, hsCRP, insulin treatment, and RAS inhibitors, baPWV was significantly correlated with the ACR (models 2 and 3; Table 3). After additional adjustments for eGFR, baPWV remained significantly associated with the ACR ($r = 0.150$, $P < 0.001$). When the patients were stratified by eGFR, baPWV was positively correlated with the ACR in the final model after adjusting for several clinical variables and eGFR in patients with an eGFR ≥ 60 mL/min/1.73 m^2 ($r = 0.146$, $P < 0.001$) and <60 mL/min/1.73 m^2 ($r = 0.091$, $P = 0.071$).

However, the significant association of baPWV with eGFR among all patients was lost after adjusting for clinical variables (models 3 to 4; Table 4). When the patients were stratified by albuminuria status, baPWV was significantly associated with eGFR in the univariate analysis (model 1; Table 4), but this significant relationship with eGFR was lost after adjusting for clinical variables in both the normoalbuminuria and albuminuria groups (models 2–4; Table 4). These results were the same when the albuminuria group was divided into the microalbuminuria group and the macroalbuminuria group (Supplementary Table 2).

4. Discussion

Although several cross-sectional studies have demonstrated that CKD is correlated with aortic stiffening [5, 7], the association of aortic stiffness with CKD in patients with diabetes has received less attention. In the present study, compared with a decline in GFR, arterial stiffness was more associated with albuminuria in patients with type 2 diabetes mellitus. However, arterial stiffness was not associated with GFR in both the normoalbuminuric and albuminuric patients with type 2 diabetes after adjusting for several significant clinical variables.

Several previous studies are in agreement with the present data regarding the relationship between arterial stiffness and the ACR and/or GFR in diabetic patients. A study of Chinese middle-aged adults demonstrated that albuminuria is strongly related to arterial stiffness (measured using baPWV) and that this relationship is enhanced in

subjects with hypertension, diabetes, or macroalbuminuria [12]. Similarly, a Japanese longitudinal study found that aortic stiffness (measured using carotid-femoral pulse wave velocity (cfPWV)) is related to incidental albuminuria and the rate of GFR decline in patients with type 2 diabetes [13]. On the other hand, pulse pressure (PP) is often used to measure arterial stiffness in the clinical field. In the Veterans Affairs Diabetes Trial (VADT) subanalysis, Anderson et al. [14] reported accelerated ACR deterioration in subjects with a relatively high PP and attenuated ACR deterioration in subjects with a relatively low PP. These authors also found that arterial stiffness (defined by a PP $\geq$ 60 mmHg) was significantly associated with a worsening ACR, but not with a worsening eGFR [14]. There is also controversy regarding the relationships between PP and ACR and/or GFR. In the Australian Diabetes, Obesity, and Lifestyle (AusDiab) study, a higher PP (defined as $\geq$61 mmHg) was a significant risk factor for a decline in eGFR, but not albuminuria, over a 5-year period, especially in individuals with type 2 diabetes [15].

The mechanisms underlying the relationship between a greater degree of arterial stiffness and heightened albuminuria or a decreased GFR in diabetic patients have not been established. As arterial stiffness increases, the myocardium and kidneys are exposed to higher systolic pressures and greater pressure fluctuations resulting in myocardial hypertrophy and fibrosis, renal microvascular damage, and an increased risk of renal dysfunction [23]. The combination of endothelial dysfunction and inflammation may be plausible mechanisms linking aortic stiffness and CKD [13]. Additionally, the afferent arteriole branch from the renal artery is a short vessel exposed to high pressures and, therefore, must maintain a strong arteriolar tone to provide a high pressure gradient over a short distance [13]. It is possible that these vessels are controlled by the hemodynamics of large arteries rather than small vessels in the peripheral circulation. As a result, the stiffness of large arteries may directly increase PP, especially in these vessels, and lead to glomerular or tubular damage via elevated intrarenal PP [23]. In turn, this may cause renal microvascular dysfunction, including albuminuria or a reduction in GFR.

One strength of the present study was the use of a large population-based cohort that included 2613 patients from eight general hospitals. Furthermore, arterial stiffness was evaluated by baPWV, which may be more applicable in general practice because its measurement is automated and easier to perform compared to that of cfPWV [24]. However, there were also several limitations to the present study. First, the present findings were based on a cross-sectional design rather than longitudinal observations. Thus, further investigations are required to find out whether patients with elevated excreted urinary markers (kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and liver-type fatty acid-binding protein (L-FABP)) and normoalbuminuria are more vulnerable to a decline in GFR or the progression of albuminuria. Our research group is now working to clear up this issue. Second, a homogeneous population was used in the present study because it was hospital based. Besides, the REBOUND study was designed to show the relationship between CVD and baPWV in patients with type 2 diabetes. Therefore, the subjects with low ABI, severe CVD, and elevated serum creatinine greater than 2.0 mg/dL, which adversely affect survival [25], were excluded. As a result, the generalization of the present findings to all patients with type 2 diabetes mellitus may be limited. Third, in order to determine urine ACRs, random spot urine samples were collected at only one time point, although urine samples were obtained from patients without illness or prior kidney diseases other than diabetic nephropathy. Fourth, it is likely that the reduction in muscle mass in elderly patients with type 2 diabetes may have influenced eGFR levels and led to a misclassification of changes in eGFR levels as well as underestimation of the relationship between aortic stiffness and decreased GFR. Fifth, the results of the present study were not novel, but it is worthwhile to show that arterial stiffness has different associations with albuminuria and GFR in a large population-based cohort.

In conclusion, the present findings demonstrated that, compared with a decrease in the GFR, arterial stiffness was more associated with albuminuria in patients with type 2 diabetes mellitus. The effect of arterial stiffening on albuminuria or a decreased GFR needs to be analyzed in future large longitudinal studies.

Conflicts of Interest

The authors declare that they have no competing interests concerning this article.

Authors' Contributions

Jong Ho Kim researched the data, contributed to the discussion, and wrote and edited the manuscript. Sang Soo Kim and In Joo Kim researched the data, contributed to the discussion, and reviewed the manuscript. Bo Hyun Kim, Ja Young Park, Chang Won Lee, Ji Hye Suk, Sun Hae Shin, Sung Pyo Son, Min Chul Kim, Jun Hyeob Ahn, Kwang Jae Lee, Min Jung Kwon, Soon Hee Lee, and Jeong Hyun Park contributed to the data collection and manuscript preparation. Sang Soo Kim and In Joo Kim are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Research Article

NGAL as an Early Predictive Marker of Diabetic Nephropathy in Children and Young Adults with Type 1 Diabetes Mellitus

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Aims. Type 1 diabetes (T1D) is often associated with early microvascular complications. Previous studies demonstrated that increased systolic (SAP) and diastolic arterial blood pressures (DAP) are linked to microvascular morbidity in T1D. The aim of the study was to investigate the predictive role of neutrophil gelatinase-associated lipocalin (NGAL) in unravelling early cardio-renal dysfunction in T1D. *Methods*. Two T1D patient groups participating in two-centre prospective cohorts were studied. Group A consisted of 57 participants aged 13.9 years (SD: 3.1) and group B consisted of 59 patients aged 28.0 years (SD: 4.4). Forty-nine healthy children [age: 10.5 years (SD: 6.6)] and 18 healthy adults [age 27.7 years (SD: 4.2)] served as controls. Serum concentrations of NGAL (ELISA) were determined, and SAP and DAP were examined (SAP and DAP also expressed as *z*-scores in the younger group). *Results*. NGAL correlated positively with SAP in both patient groups ($P = 0.020$ and $P = 0.031$, resp.) and SAP *z*-score ($P = 0.009$) (group A) and negatively with eGFR in both groups ($P < 0.001$ and $P < 0.001$, resp.). *Conclusions*. NGAL may be proposed as a biomarker of early renal dysfunction even in nonalbuminuric T1D patients, since it was strongly associated with renal function decline and increasing systolic arterial pressure even at prehypertensive range in people with T1D, in a broad age range.

1. Introduction

Type 1 diabetes mellitus (T1D) is a prevalent autoimmune disease in childhood and young adulthood. Diabetes nephropathy (DN) is a chronic devastating complication associated with an increased risk of end-stage renal failure, as well as cardiovascular disease and premature death. It has been previously reported that childhood-onset T1D is associated with a 4-fold increase in the overall standardized mortality rate [1].

In both the USA and Europe, approximately 20% of T1D persons develop DN and progress to end-stage renal disease (ESRD). Since T1D often occurs in younger ages, ESRD most often develops at an earlier age, during the most productive years of persons with the disease. Thus, it represents a significant burden to the patients and the society they live in [2, 3]. Moreover, nonalbuminuric DN has been reported to have a prevalence of 2% among people with T1D and chronic kidney disease (CKD) [4, 5].

Nowadays, the screening of DN is based on microalbuminuria (MA) assessment [6], and MA may be found in 12–16% of adolescents with T1D [7]. In early stages, regression to nonalbuminuria is frequently observed [8]. Puberty itself, as well as poor glycaemic control, is an independent risk factor for MA in persons with T1D [9]. However, the diagnostic value of microalbuminuria in DN has recently been challenged by a large number of researchers worldwide, while it has been widely proposed that other biomarkers are needed for the early identification of diabetic renal lesions [2, 5, 10, 11]. Previous studies have provided evidence of several clinical

and laboratory predictors for DN, such as increased systolic arterial blood pressure (SAP), even within the prehypertensive range [12], and dyslipidaemia, identifying people at risk for early endotheliopathy and cardiovascular disease (CVD) as well [13]. Among T1D individuals with nephropathy and hypertension, 50% will progress to end-stage renal disease within a decade [6].

The pathophysiologic changes in DN linked to renal function decline are associated with cellular and extracellular derangements in both the glomerular and tubular compartments [14]. Several studies have reported that nonalbuminuric subjects, including prepubertal children with long-standing diabetes, often have glomerular basement membrane (GBM) thickening, mesangial expansion [15, 16], and significant glomerulopathy lesions [17, 18]. Glomerular and renal tubular interstitial injury plays a role in the pathogenesis of DN [19], and various tubular markers have been assessed in the early detection of DN [10, 20]. Among them, neutrophil gelatinase-associated lipocalin (NGAL), first purified and identified in 1993 by Kjeldsen et al., seems to be a promising biomarker [19, 21]. NGAL is a 178-amino acid 25 kDa protein that belongs to the lipocalin protein family. It is primarily produced in renal tubules in response to structural kidney injury [22].

In contrast to conventional markers, such as serum creatinine, blood urea nitrogen, or serum cystatin C (CysC), NGAL is not considered a marker of renal function, but rather reflects structural damage of renal cells. In previous studies, NGAL was reported as effective in the early diagnosis of acute kidney injury (AKI) in several clinical settings [23–25] and was also validated as a significant prognostic factor in cardiovascular morbidity [26]. The association between the early tubular lesions in nonalbuminuric patients with T1D and NGAL was further supported by recently published studies [18, 19, 21].

The aim of this study was to determine the possible predictive role of serum NGAL as a supplementary marker to urinary albumin excretion, in unmasking early renal structural injury, renal function decline, and cardiovascular risk in asymptomatic, normotensive individuals in two different age groups, childhood and adulthood, with various duration of T1D and irrespective of the presence of microalbuminuria.

2. Materials and Methods

Two observational cross-sectional prospective long-term follow-up studies took place in two university diabetes centres.

The group A consisted of 57 participants with T1D with a mean age of 13.9 years (SD: 3.1) and a mean diabetes duration of 5.4 years (SD: 3.3) at the time of the evaluation, who were prospectively followed at the Diabetes Centre of the First Department of Pediatrics of the University of Athens, Greece (Table 1) [18].

The group B consisted of 59 young adults with T1D with a mean age of 28.0 years (SD: 4.4) and a mean diabetes duration of 7.4 years (SD: 1.9) at the time of the evaluation, who were prospectively followed for at least 5 years at the Department of Endocrinology of the University Hospital of Linkoping, Sweden (Table 1) [27]. The diagnosis of T1D was based in all participating patients on the presence of the positive titer of at least one of the known circulating, islet-specific, pancreatic autoantibodies related to T1D (autoantibodies against glutamic acid decarboxylase); the 40 K fragment of tyrosine phosphatase (IA2); or insulin antibodies. Regarding group A, eight of the patients presented with microalbuminuria at inclusion in the study, while five of them had known persistent microalbuminuria. In group B, seven out of 59 patients had persistent microalbuminuria at enrolment.

Forty-nine healthy children with a mean age of 10.57 (SD: 6.6) served as controls (group C) (Table 1). Informed consent was obtained from all the participants and their parents before their inclusion in the study. Eighteen healthy adults with a mean age of 27.7 (SD: 4.2) also served as controls (group D).

Both studies were approved by the respective Ethics Committee (Ethics Committee of the Aghia Sophia Children's Hospital in Athens, Greece, and Ethics Committee of the Medical Faculty in Linkoping, Sweden, resp.).

Inclusion criteria for all participants were T1D while exclusion criteria were the presence of active urinary tract infection, glucocorticoid medication, antihypertensive treatment, pregnancy, renal disease, and any chronic disease besides T1D.

Estimated glomerular filtration rate (eGFR) was calculated using the most recently suggested formula by Grubb et al. ($\text{eGFR} = 130 \times \text{cystatin C}^{-1.069} \times \text{age}^{-0.117} - 7$) [28].

Serum and urinary NGAL levels were measured using a commercially available ELISA (Bioporto, Gentofte, Denmark). The reference range in plasma was 37–106 ng/ml, and the intra- and interassay coefficients of variation were 5.6% and 6.4%, respectively.

Cystatin C concentration was determined by an immunonephelometric technique using the BN Prospec nephelometer (Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany). The interassay coefficient of variation for the assay was 5.05% and 4.87% at mean concentrations of 0.97 and 1.90 mg/l, respectively, and the reference range in plasma was 0.47–1.09 mg/l.

Statistical analyses were performed using MedCalc, version 12.5 (MedCalc Software, Ostend, Belgium). Correlation analysis was used to determine whether the values of the two variables are associated using Pearson parametric correlation. Student *t*-test was performed (paired samples *t*-test was used in order to test the null hypothesis that the average of the deviations between a series of paired observations is zero when carried out on the same individuals or independent samples *t*-test when performed between the patient and control groups). Multiple regression analysis was used to test the relation between one dependent variable and at least one independent variables. The significance was defined as a P value < 0.05, rho, and 95% confidence interval (CI) for the correlation coefficient.

3. Results

The mean value for NGAL in group A was 67.6 ng/ml (SD: 27.9) while in group B, it was 85.2 ng/ml (SD: 29.4)

Table 1: Background data, clinical examination, and biochemical measurement results for 57 children with type 1 diabetes, 59 adult patients with type 1 diabetes, and healthy control participants.

Variable	Type 1 diabetes children group (group A)	Healthy control children group (group C)	*P* value	Type 1 diabetes adult group (group B)	Healthy control adult group (group D)	*P* value
Gender	Male 32; female 25	Male 28; female 21		Male 34; female 25	Male 8; female 10	
Age (years)	13.9 ± 1.9 (3.5–18)	10.5 ± 3.3 (3–18)		28.0 ± 2.2 (20–35)	27.7 ± 2.6 (21–36)	
Age at onset (years)	8.5 ± 3.4 (2–15)	NA	NA	8.6 ± 4.1 (1–16)	NA	NA
Diabetes duration (years)	5.4 ± 1.8 (1–14.5)	NA	NA	7.4 ± 0.9 (4.8-9.4)	NA	NA
Mean HbA1c (95% CI for the mean)	7.88%–8.85% (5.8%–14%) or 62.6–73.2 mmol/mol (39.9–129.5 mmol/ mol)	4.4%–4.9% or 25–30 mmol/mol	$P < 0.001$	7.06%–7.88% (4.6%–11.7%) or 53.7–62.6 mmol/mol (26.8–104.4 mmol/ mol)	4.1%–5.2% or 23–33 mmol/ mol	$P < 0.001$
BMI (95% CI for the mean)	NA	NA		25.0–27.3 (20.1–40.4)	25.9–30.6 (21.0–30.4)	
BMI *z*-score (95% CI for the mean)	0.2 to 0.8 (−1.0–1.5)	−0.1 to 0.7 (−1.43–1.11)		−0.2 to 0.2 (−1.4–3.3)	−0.1 to 1.2 (−1.3–2.3)	NA
Systolic arterial pressure (mmHg) (95% CI for the mean)	109.81 to 117.18 (76–133)	98.24 to 118.75 (90–119)	$P = 0.55$	121.44 to 126.33 (108–140)	105.35 to 116.31 (100–140)	$P = 0.272$
Systolic arterial pressure *z*-score (95% CI for the mean)	0.02 to 0.58 (−0.9–2.8)	0.01 to 0.39 (−0.4–2.5)	$P = 0.19$	NA	NA	NA
Diastolic arterial pressure (mmHg) (95% CI for the mean)	65.3 to 70.5 (50–85)	56.9 to 67.0 (51–75)	$P = 0.007$	75.8 to 80.0 (60–98)	66.7 to 76.0 (55–90)	$P = 0.33$
Microalbuminuria	8 out of 57	None		7 out of 59	None	
Diastolic arterial pressure *z*-score (95% CI for the mean)	0.14 to 0.5 (−1.1–1.9)	−0.9–0.2 (−1.6–1.5)	$P = 0.023$	NA	NA	NA
NGAL (ng/ml) (95% CI for the mean)	60.2 to 75.0 (22.7–190.3)	20.1 to 29.2 (5.1 to 81.4)	$P < 0.001$	77.0 to 93.4 (39.6–186.5)	67.4 to 84.9 (47.7–109.7)	$P = 0.039$
Cystatin C (mg/l) (95% CI for the mean)	0.23 to 0.92 (0.62 to 0.68)	0.37 to 0.84 (0.63 to 0.78)	$P = 0.53$	0.52 to 1.06 (0.66 to 0.81)	0.38–1.30 (0.68 to 0.75)	$P = 0.68$
eGFR (ml/min/1.73 m^2) (95% CI for the mean)	98.6 to 105.9 (84.4–151.3)	89.0 to 107.4 (76.4–141.1)	$P = 0.70$	56.6 to 243.3 (113.4–129.5)	72.8 to 170.9 (103.7 to 131.3)	$P = 0.49$

NA: not applicable.

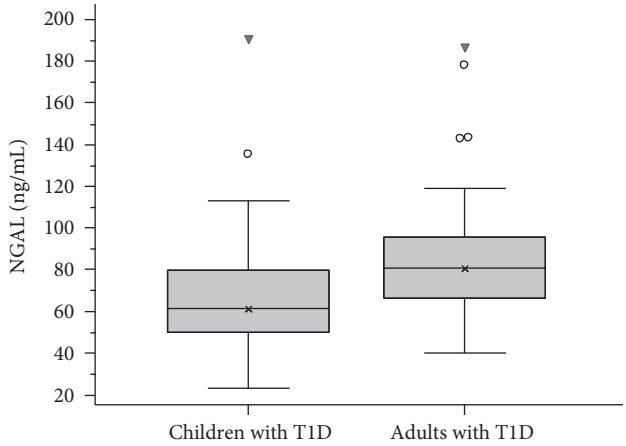

Figure 1: Box plot of NGAL levels in children and adults with T1D. Boxes represent the interquartile range; lines inside the boxes represent the median value; cross represents mean marker; and whiskers represent the lowest and highest observations, respectively. Children group had statistically significant lower median value than the adult group ($P < 0.001$).

($P < 0.001$) (Figure 1). Both groups had a significantly higher mean value compared with the two control groups [mean NGAL: 24.6 ng/ml (SD: 15.8) for group C and 76.1 ng/ml (SD: 19.2) for group D], according to paired t-test analysis for independent groups ($P < 0.001$ and $P = 0.039$, resp.) (Figure 2).

The mean value for cystatin C in group A was 0.65 ng/ml (SD: 0.1) while in group B, it was 0.71 mg/l (SD: 0.1). Both groups had no significantly higher mean value compared with the two control groups [mean cystatin C: 0.68 ng/ml (SD: 0.1) for group C and 0.74 ng/ml (SD: 0.02) for group D] per paired t-test analysis for independent groups.

Regression analysis revealed that NGAL had a negative correlation with eGFR in both group A ($r = -0.501$, $P < 0.001$) and group B ($r = -0,418$, $P < 0.001$). Regression analysis of NGAL levels and eGFR values, adjusted for age in both groups A and B with type 1 diabetes, showed a significant correlation between the biomarker and renal function decline (F ratio = 5.93, $P = 0.0037$) (Figure 3). No significant correlation was found between NGAL and eGFR in either control groups.

NGAL was positively correlated with systolic arterial pressure, according to logarithmic curve regression equations in both patient groups ($P = 0.020$ for group A and $P = 0.031$ for group B). In children group A, SAP z-score was also analysed and a positive correlation with NGAL was revealed ($P = 0.009$). Regression analysis adjusted for age showed a significant positive correlation of NGAL with systolic arterial pressure in both groups with type 1 diabetes (F ratio = 17.1, $P = 0.0001$) (Figure 4). No significant correlation between NGAL and either DAP or DAP z-score was noted. NGAL was not significantly correlated with SAP, SAP z-score, DAP, or DAP z-score in the control groups.

Urinary NGAL was analysed, but no significant correlation with neither SAP nor eGFR was revealed.

Neither microalbuminuria nor HbA1c had any significant correlations with NGAL in either group.

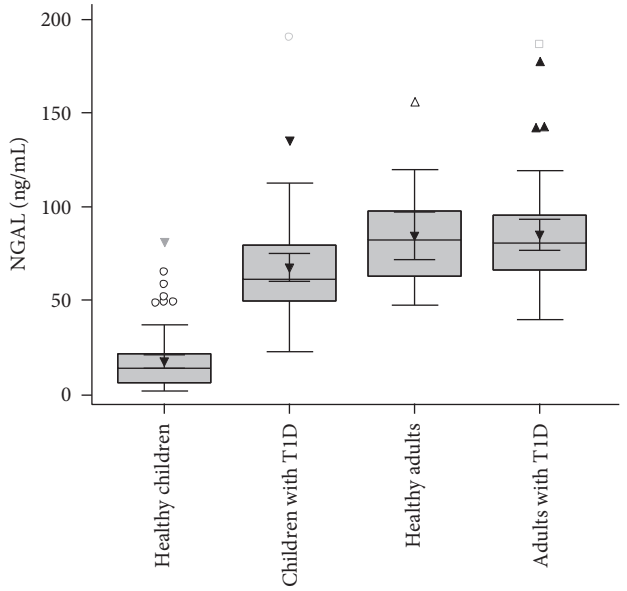

Figure 2: Box plot of NGAL levels. Boxes represent the interquartile range; lines inside the boxes represent the median value; cross represents mean marker; and whiskers represent the lowest and highest observations, respectively. The mean value for NGAL in children with T1D was 67.67 ng/ml (SD: 27.93) while in the group of adults with T1D, it was 85.26 ng/ml (SD: 29.49). Both groups had a significantly higher mean value compared with the two control groups (mean NGAL: 24.69 ng/ml (SD: 15.89) for the children control group ($P < 0.001$) and 76.18 ng/ml (SD: 19.22) for the adult control group ($P = 0.039$)) according to paired t-test analysis for independent samples.

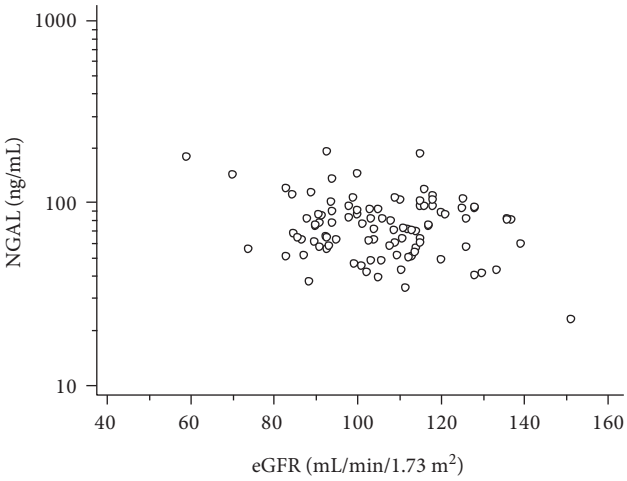

Figure 3: Serum NGAL had a negative correlation with eGFR values according to regression analysis, adjusted for age in both children and adult groups with type 1 diabetes (F ratio = 5.93, $P = 0.0037$).

4. Discussion

Microalbuminuria has been considered the earliest marker of the development of diabetes nephropathy (DN) and is often linked to established significant glomerular damage,

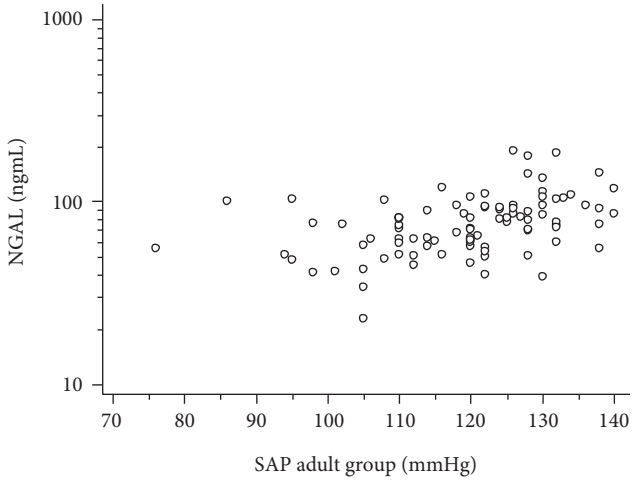

Figure 4: Serum NGAL had a significant positive correlation with systolic arterial pressure for children and adult groups with type 1 diabetes according to regression analysis adjusted for age (F ratio = 17.1, $P = 0.0001$).

traditionally believed to be the most common lesion in type 1 DN. However, recent studies showed that MA might be transient and not necessarily reflect permanent renal impairment [8]. Further recent reports suggested that pathophysiologic changes in DN include renal function decline associated with both cellular and extracellular derangements in glomerulotubular compartments [17, 18]. Besides, several lines of evidence suggest that early lesions in both glomerular and tubular structures may be present in nonalbuminuric patients. In line with this, cohort studies, including prepubertal children with average diabetes duration of 5–8 years, revealed glomerular base membrane thickening and mesangial expansion [16], while they also disclosed that longstanding nonalbuminuric T1D participants might have significant glomerulopathy lesions [17–19, 21]. Moreover, the prevalence of nonalbuminuric CKD in T1D was recently reported to be 2% and was associated with a higher risk of cardiovascular morbidity as well as all-cause mortality in people with T1D [4, 5].

The increased hyperglycaemia-induced permeability of the glomeruli that is associated with hyperfiltration and microalbuminuria is not debated, but the perception of microalbuminuria being the only marker for diagnosing and excluding the development of diabetic nephropathy needs to be further investigated, to reduce the number of the young T1D normoalbuminuric people who will progress to CKD. According to the National Kidney Foundation (NKF), a patient is considered to be diagnosed with chronic kidney disease (CKD) if he or she presents a GFR < 60 ml/min for three months or more. Alternatively, other ongoing structural or functional renal abnormalities, which can be detected by pathological abnormalities or specific markers, are considered CKD [29]. Besides, early renal function decline, defined as a progressive loss of GFR over time even if it remains within the normal range, is also supported to be associated with DN in T1D [30].

Our study revealed strong negative correlations between serum NGAL and eGFR in both adult and children groups, indicating a significant association with renal function decline. No significant associations between urinary NGAL and eGFR were found in this study, and this finding does not agree with previous reports.

This finding underpins the value of NGAL as a biomarker of early renal damage in T1D, since NGAL had a positive correlation with the duration of T1D, mainly when eGFR was decreased, and this finding may reflect a progress of the early renal structural damage occurring during the disease, even if eGFR remains within the normal range. This finding is probably suggestive that NGAL is associated with the occurrence of ERFD. The number of the participants with T1D who presented microalbuminuria was much lower than the number of patients who presented a decreased eGFR. Moreover, the fact that a percentage of nonalbuminuric patients, who were found to have renal function decline, had higher NGAL levels, while some patients with microalbuminuria had normal NGAL values, may suggest that these two biomarkers, that is, microalbuminuria and NGAL, may reflect other sites of renal injury during the process of DN establishment. The link between the early tubular damage in nonalbuminuric people with T1D and increased NGAL is further supported by recently published studies [15, 17]. The mean value of NGAL was significantly higher in the adult patient group. Besides, regression analysis revealed a correlation between age and the duration of type 1 diabetes in both children and adult. Urinary NGAL was also analysed in the paediatric participants, but no significant correlation with either GFR or SAP was found, and this finding does not agree with previous reports [31].

Systolic arterial pressure has been previously suggested as a predictor of DN [3]. In our study, since it was particularly focused on a young population, we estimated both SAP z-score and SAP for children and SAP for adults. We found that in two different age groups with T1D of various disease durations, NGAL correlated positively with increasing systolic arterial pressure, even if the latter remained within the prehypertensive or normal range. It has been previously reported that ambulatory blood pressure modestly rising in T1D patients is associated with the silent phase of DN up to 5 years before microalbuminuria appears [13]. Also, past studies had shown an association between renohypertensive diseases and NGAL [31]. Our findings may suggest that the association between NGAL and increasing SAP could reflect the early asymptomatic stages of DN progression in T1D and unravel possible underlying early renal injury. Since we found no similar significant associations in the control groups, our hypothesis is that the findings revealed from the two patient groups reflect the underlying microvascular pathogenesis in type 1 diabetes. Diabetic nephropathy has been linked with biomarker changes consistent with generalized endothelial dysfunction. Markers of endothelial dysfunction and arterial stiffness have also been correlated with MA in diabetic populations [16]. The association between NGAL and increasing SAP may suggest an indirect predictive role of NGAL as a cardiovascular morbidity marker. Undoubtedly, further studies investigating the endothelial dysfunction will further delineate the extent of microvascular damage in DN.

5. Conclusions

To our knowledge, this is the first study that aimed at assessing the predictive value of other early markers of renal injury, besides microalbuminuria, such as NGAL in two different age groups of patients with T1D and found similar results. Moreover, a predictive role of NGAL has been demonstrated, as an early marker of diabetes nephropathy and probably asymptomatic cardiovascular morbidity independent of microalbuminuria.

Defining new predictive markers as supplementary tests to urinary albumin excretion for the early diagnosis of microvascular complications in T1D would lead to effective management and treatment approaches in time, which are needed to minimize the rates of severe reno- and cardiovascular as well as all-cause-associated morbidity in young people with T1D. Therefore, these data need to be further confirmed by large-scale longitudinal studies before being integrated into the risk assessment of follow-up of people with T1D.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

The *CNDP1* $(CTG)_5$ Polymorphism Is Associated with Biopsy-Proven Diabetic Nephropathy, Time on Hemodialysis, and Diabetes Duration

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Considering that the homozygous *CNDP1* $(CTG)_5$ genotype affords protection against diabetic nephropathy (DN) in female patients with type 2 diabetes, this study assessed if this association remains gender-specific when applying clinical inclusion criteria (CIC-DN) or biopsy proof (BP-DN). Additionally, it assessed if the prevalence of the protective genotype changes with diabetes duration and time on hemodialysis and if this occurs in association with serum carnosinase (CN-1) activity. Whereas the distribution of the $(CTG)_5$ homozygous genotype in the no-DN and CIC-DN patients was comparable, a lower frequency was found in the BP-DN patients, particularly in females. We observed a significant trend towards high frequencies of the $(CTG)_5$ homozygous genotype with increased time on dialysis. This was also observed for diabetes duration but only reached significance when both $(CTG)_5$ homo- and heterozygous patients were included. CN-1 activity negatively correlated with time on hemodialysis and was lower in $(CTG)_5$ homozygous patients. The latter remained significant in female subjects after gender stratification. We confirm the association between the *CNDP1* genotype and DN to be likely gender-specific. Although our data also suggest that $(CTG)_5$ homozygous patients may have a survival advantage on dialysis and in diabetes, this hypothesis needs to be confirmed in a prospective cohort study.

1. Introduction

Diabetic nephropathy (DN) occurs in approximately 40% of patients with type 1 and type 2 diabetes [1] and is the leading cause of end-stage renal disease (ESRD) [2]. Compelling evidence has shown that susceptibility to DN is genetically determined [3, 4]. Amongst the reported linkage studies, there seems to be consistency in the linkage between human chromosome 18q22.3-q23 and DN [4–7]; linkage to the DN trait on chromosomes 7q21.3, 10p15.3, and 14q23.1 has also been reported [7]. Linkage with 18q22.3 was observed in populations of different ethnicities, for example, American Indians [8], Afro-Americans [9], and Caucasians [5].

Janssen et al. initially postulated that the *CNDP1* gene on chromosome 18q22.3-q23, encoding serum carnosinase (CN-1), is a susceptibility gene for DN in type 2 diabetes mellitus (T2DM) patients [10]. It was found that T2DM patients homozygous for the *CNDP1* $(CTG)_5$ allele are less

frequently affected by DN compared to T2DM patients with other *CNDP1* genotypes [10]. The prevalence of the $(CTG)_5$ allele strongly varies with different ethnicities. While homozygosity for the $(CTG)_5$ allele is more frequent in the European population (38.6% in healthy controls and 29.3% in diabetic patients with ESRD) [11], this genotype seems to be much more rare in the Asian population with a high prevalence of DN [12, 13]. It has also been reported that the association between the *CNDP1* genotype and DN is sex-specific and independent of susceptibility to T2DM [14].

Since most patients with T2DM are not formally evaluated with a renal biopsy, the diagnosis of DN is based on clinical criteria, for example, persistent macroalbuminuria on at least 2 independent occasions (albumin excretion rate > 300 mg/d or >200 mg/l or ACR (albumin/creatinine ratio) > 300 mg/g). Yet, biopsy-based retrospective evaluations of the prevalence of nondiabetic renal disease (NDRD) in T2DM patients revealed a high percentage of patients having NDRD without evidence of concurrent DN [15, 16]. Although the predictive value of clinical criteria for DN in T2DM patients can be improved by the presence of proliferative retinopathy [17, 18], genetic studies that use clinical inclusion criteria (CIC) for group allocation still bear the risk of wrongly assigning patients to the DN group. In the present study, we assessed if the association between *CNDP1* and DN is still observed when applying CIC or biopsy-proven diabetic nephropathy (BP-DN) and, if so, whether this is only observed in female T2DM patients. Since also an association between the *CNDP1* $(CTG)_5$ homozygous genotype and cardiovascular mortality has been reported to be sex-specific [19], we also assessed if the prevalence of this genotype changes with diabetes duration and time on dialysis. Since T2DM patients on dialysis have a high mortality risk, it would be expected that the proportion of *CNDP1* $(CTG)_5$ homozygous patients would decrease with time on dialysis, particularly in females.

2. Materials and Methods

2.1. Patients. Patients were recruited between 2011 and 2014 from the Fifth Medical Clinic and Dialysis Unit at the University Medical Centre Mannheim and different nephrology practices in proximity (Centre for Renal Disease, Weinheim, Lindenfels, Viernheim; Nephrocare Ludwigshafen GmbH; KfH Nierenzentrum, Ludwigshafen; Nephrology Practice Frankenthal, Bad Dürkheim, Lampertheim). After screening of clinical records ($n = 384$) and patients with a biopsy-proven renal diagnosis ($n = 52$), a total of 436 patients were deemed to be eligible for this study. Due to the retrospective nature of this study, indications for renal biopsies were not uniform and the histological evaluation was undertaken by different pathologists. Out of the 436 initially selected patients, 66 were excluded because of incomplete clinical data or missing informed consent. The remaining patients ($n = 370$) were allocated to 5 different groups, that is, 130 T2DM patients without DN (no-DN), 108 T2DM patients with CIC-DN, 30 T2DM patients with BP-DN, 80 patients with CIC nondiabetic renal disease (CIC-NDRD), and 22 patients with biopsy-proven nondiabetic renal disease (BP-NDRD) (Figure 1). Patients without renal biopsy material were allocated on the basis of clinical criteria as specified below, whereas patients with renal biopsy material were allocated either to the BP-DN or to the BP-NDRD group. The ethnicity distributions in the no-DN and BP-DN groups were broadly comparable (no-DN: 75% from Germany, 12.5% from Turkey; BP-DN: 70% from Germany, 17% from Turkey).

Inclusion criteria for clinical diagnosis of DN were as follows: persistent macroalbuminuria on at least 2 independent occasions (albumin excretion rate > 300 mg/d or >200 mg/l or ACR (albumin/creatinine ratio) > 300 mg/g) in combination with the diagnosis of diabetic retinopathy (DR) (all severity degrees were allowed). This combination was obligatory to reduce the possibility that cases with proteinuria due to renal disease other than DN (NDRD) were included [20]. Anuric patients with a history of macroalbuminuria were included. Exclusion criteria were urinary tract infection or fever at the time of urine investigation, documented renal disease other than DN, and a history of kidney transplantation.

T2DM patients without DN (=control group) fulfilled the following criteria: diabetes duration of at least 15 years accompanied by normoalbuminuria on at least two independent occasions (albumin excretion rate < 30 mg/d or <20 mg/l or ACR< 30 mg/g). Since approximately 80% of the diabetic patients in this group were on ACE inhibitors or AT_1 blockers, false-negative results based on albuminuria could not be excluded. However, to minimize the possibility of DN patients in the control group, only the normoalbuminuric patients with no or mild nonproliferate DR were included since the presence of nephropathy without significant DR is rare [18, 21, 22]. Diabetes mellitus was defined by a documented history of diabetes or a fasting blood glucose of ≥7.0 mmol/l (126 mg/dl), a casual plasma glucose level of ≥11.1 mmol/l (200 mg/dl), or a HbA1c level of ≥6.5%.

Nondiabetic patients with ESRD were included in the CIC-NDRD group ($n = 80$) after screening of patient records and medications along with laboratory testing for plasma glucose or HbA1c levels to exclude diabetes.

Out of the whole cohort, 175 patients were on hemodialysis including 90 diabetic patients and 85 NDRD patients. Patients were dialyzed using a blood flow rate of 200–300 ml/min and a dialysate flow rate of approximately 500 ml/min.

Estimated GFR was calculated based on the MDRD formula [23]. Serum was used to measure CN-1 activity and concentration. Genotyping was performed on EDTA blood. All samples were stored at −20°C until use. The study protocol was approved by the local ethics committee, and all patients gave written informed consent prior to the study enrollment (no. 0193/2001).

2.2. Genotyping. Genomic DNA was isolated from whole blood using the Genomic DNA isolation kit (Promega, Mannheim, Germany) according to the manufacturer's instruction and stored at −20°C until use. A 167-base pair fragment spanning the $(CTG)_n$ polymorphism of *CNDP1* was amplified by standard PCR methods using a fluorescence

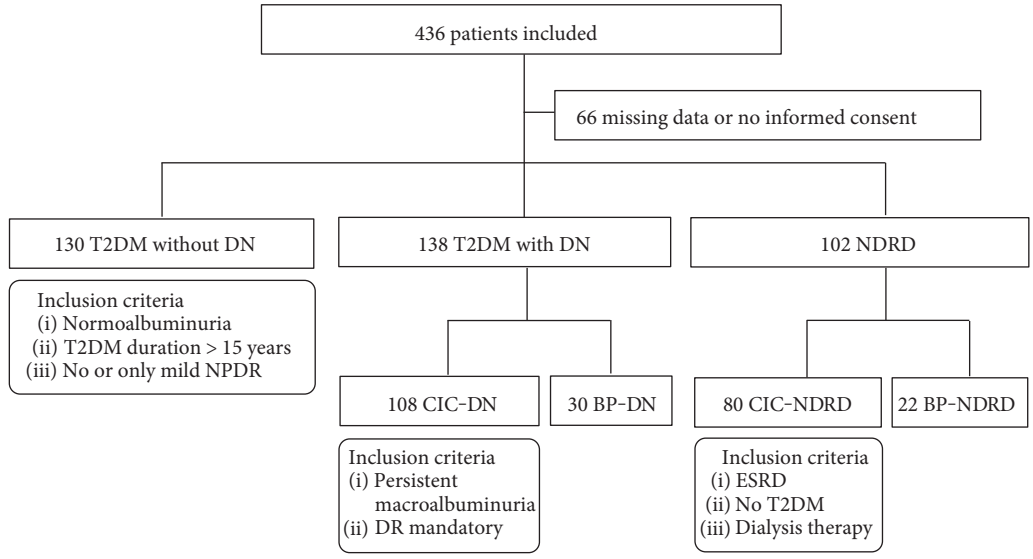

Figure 1: Flow diagram for patient recruitment and group allocation. DN: diabetic nephropathy, NDRD: nondiabetic renal disease, NPDR: nonproliferative diabetic retinopathy, CIC: clinical inclusion criteria, BP: biopsy-proven, DR: diabetic retinopathy, ESRD: end-stage renal disease.

labeled forward primer (5′FAM-AGGCAGCTGTGTGAGG TAAC-3′) and an unlabeled reverse primer (5′-GGGTGAG GAGAACATGCC-3′), respectively. Genotyping was performed by means of fragment analysis on an ABI 310 sequencing platform (ABI PRISM DNA analyzer 3100).

2.3. CN-1 Activity Assay. CN-1 activity was assayed based on the method described by Teufel et al. [24].

2.4. Statistical Analysis. Quantitative data are depicted as median with corresponding 25th and 75th percentiles (interquartile range) or, when appropriate, as mean ± SEM. Student's *t*-test was carried out for comparison of continuous variables. Qualitative data were analyzed using the χ^2 test. For pairwise comparisons, frequency tables were partitioned into respective 2×2 subtables. The significance level was corrected using the Bonferroni method based on the number of planned comparisons. Univariate and multivariate logistic regression analyses were performed to examine predictors of biopsy-proven diabetic nephropathy. Variables with a P value of <0.25 in the univariate analysis were included into a full-model multivariate analysis. To compare frequencies among groups, which have an ordering, the χ^2 test for trend (Cochran-Armitage test for trend) was carried out. Time on hemodialysis was logarithmically transformed before the correlation analysis because of its skewed distribution. The significance level α was defined as 0.05. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, California) and Microsoft Excel/XLSTAT 19.01 (Addinsoft, New York, USA).

3. Results

3.1. Patient Characteristics. Demographic and clinical characteristics of all studied individuals are presented in Table 1. Significantly more male patients (64%) were recruited in the CIC-DN group (males: $n = 69$, females: $n = 39$) as compared to the no-DN group (50%) (males: $n = 65$, females: $n = 65$). Gender distribution in the other groups resembled the CIC-DN group (CIC-NDRD: 61% males, BP-DN: 70% males and BP-NDRD: 64% males). BP-NDRD patients were diagnosed with hypertensive nephrosclerosis, IgA nephropathy, lupus nephritis, or other categories of glomerulonephritis (e.g., granulomatosis with polyangiitis and minimal change GN). Irrespective of subgroup analyses, the distribution of the most prevalent ethnicities was comparable in the no-DN and BP-DN groups as determined by means of χ^2 test. Also, the frequency of $(CTG)_5$ homozygosity did not differ between the two major ethnicities, German and Turkish.

It should be noted that in the group of BP-DN, DM duration was shorter (time from DM diagnosis: 14 (9–20) years versus 21.0 (15–29) years, BP-DN versus CIC-DN) with more severe hyperglycemia and albuminuria (HbA1c: 7.6 (6.7–8.8) % versus 7.3 (6.8–8.2) %, albuminuria: 2070 (337–3290) mg/l versus 644 (327–2110) mg/l, BP-DN versus CIC-DN).

3.2. Association of the CNDP1 $(CTG)_5$ Homozygous Genotype with Diabetic Nephropathy. If diagnosis of DN was based on CIC alone, the frequency of the homozygous $(CTG)_5$ genotype did not significantly differ between the no-DN and CIC-DN groups (Figure 2(a), 36% versus 38%). The frequency of the protective genotype dropped to 17% when biopsy-proven DN was considered only (36% versus 17%; no-DN versus BP-DN, $P < 0.05$, NS after Bonferroni correction) (Figure 2(a)).

To confirm the previous findings of the sex-specific association between DN and the homozygous $(CTG)_5$ genotype, patients were stratified according to gender. In male patients, neither CIC-DN nor BP-DN was associated with $(CTG)_5$ homozygosity when compared to no-DN (no-DN: 34%, CIC-DN: 42%, BP-DN: 24%) (Figure 2(b)). Although the

Table 1: Demographic and clinical data of all patients.

	No-DN	CIC-DN	BP-DN	CIC-NDRD	BP-NDRD
N	130	108	30	80	22
Demographic characteristics					
Male sex—*n* (%)	65 (50)	69 (64)	21 (70)	49 (61)	14 (64)
Age—year	71 (63–75)	71 (62–76)	61 (57–69)	61 (48–74)	61 (55–78)
Clinical characteristics					
Body mass index—kg/m^2	31.1 (28–35)	29.8 (27–35)	29.9 (25–35)	24.5 (21–27)	27 (24–32)
Hypertension					
Number of AHM	3 (2–3)	3 (2–4)	3 (2–5)	3 (1.5–4)	2 (2–4)
Blood pressure—mmHg					
Systolic	129 (120–140)	135 (120–156)	140 (130–150)	135 (120–145)	140 (128–153)
Diastolic	70 (66–80)	70 (60–80)	75 (70–80)	70 (60–80)	70 (64–80)
Diabetes mellitus					
Time from diagnosis—year	16 (13–20)	21 (15–29)	14 (9–20)	—	—
HbA1c—%	7.0 (6.4–8.1)	7.3 (6.8–8.2)	7.6 (6.7–8.8)	5.6 (5.4–5.7)	5.6 (5.1–6.2)
Kidney function					
Creatinine—mg/dl	0.9 (0.8–1.1)	6.3 (3.7–8.8)	5.7 (3.0–7.3)	9.7 (7.5–11.5)	3.4 (1.7–5.0)
eGFR—ml/min	73 (61–87)	9 (6–16)	10 (6–20)	5 (4–8)	15 (8–37)
Hemodialysis—*n* (%)	0 (0)	83 (75)	18 (60)	85 (100)	8 (36)
HD duration—months*	0 (0–0)	26 (1–69)	3 (0–30)	56 (28–100)	0 (0–0.3)
Albuminuria—mg/l	9 (5–16)	644 (327–2110)	2070 (337–3290)	470 (261–1587)	556 (189–1308)
Retinopathy (*DR*)—*n* (%)					
No DR	107 (82)	1 (0)	6 (20)	—	—
NPDR	23 (18)	68 (63)	11 (37)	—	—
Proliferative DR	—	17 (16)	4 (13)	—	—
Maculopathy	—	8 (7)	3 (10)	—	—
Laser therapy	—	13 (12)	3 (10)	—	—
Polyneuropathy—*n* (%)	54 (42)	56 (52)	17 (57)	—	—
History—n (%)					
Coronary heart disease	42 (32)	74 (69)	11 (37)	24 (30)	6 (22)
Cardiovascular event	20 (15)	42 (39)	7 (23)	14 (18)	5 (23)
Arterial occlusive disease	24 (18)	56 (52)	11 (37)	17 (21)	3 (14)
Stroke	19 (15)	28 (26)	6 (20)	8 (10)	2 (9)
Statin	86 (66)	79 (73)	19 (63)	28 (35)	10 (45)
Homozygous CTG_5—*n* (%)	47 (36)	41 (38)	5 (17)	32 (40)	7 (32)

*Patients on hemodialysis only. Categorical data are represented as numbers (%) and continuous data as median with corresponding 25th and 75th percentiles (IQR). AHM: antihypertensive medication; eGFR: estimated glomerular filtration rate; HD: hemodialysis; DR: diabetic retinopathy; NPDR: nonproliferative diabetic retinopathy.

homozygous $(CTG)_5$ genotype was less frequent in both the female CIC-DN and the female BP-DN groups as compared to the no-DN group, this difference was only significant for BP-DN (38% versus 0%, no-DN versus BP-DN, $P < 0.05$, significant after Bonferroni correction) (Figure 2(c)).

To confirm that $(CTG)_5$ homozygosity is an independent, negative predictor for biopsy-proven diabetic nephropathy, multivariate logistic regression analysis was performed (Table 2). Seven diabetes-associated factors were selected as independent variables (age, BMI, diabetes duration, HbA1c, male sex, systolic blood pressure, and $(CTG)_5$ homozygosity). All variables except BMI showed a P value below 0.25 in univariate analysis and were consequently included in the multivariate model. The Hosmer-Lemeshow test demonstrated an excellent goodness of fit ($\chi^2 = 3949$, $P = 0.862$) of the resulting multivariate model. The area under the receiver operating characteristic curve (ROC-AUC) further indicated adequate discrimination (AUC = 0.797).

The $(CTG)_5$ homozygous genotype was significantly associated with biopsy-proven nephropathy in both univariate (OR = 0.353, $P = 0.047$) and multivariate analyses (OR = 0.307, $P = 0.046$) and, as such, identified as an independent protective factor. Interestingly, significance was reached despite the markedly lower number of cases ($n = 30$) generally

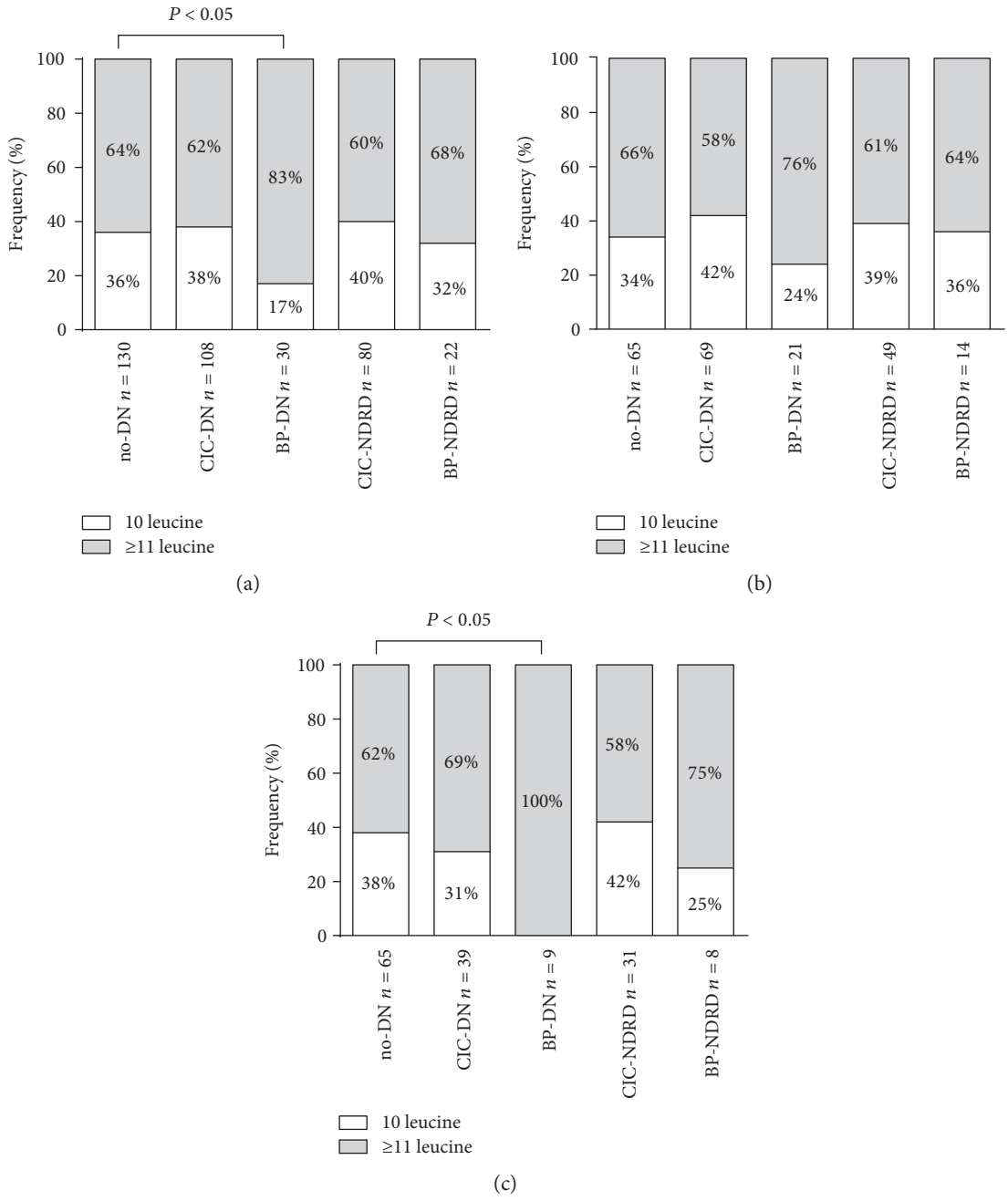

Figure 2: *CNDP1* $(CTG)_n$ genotype distribution in T2DM patients. Genotype distribution is depicted as homozygosity for the $(CTG)_5$ allele (10 leucine) versus all other genotypes (≥11 leucine). Planned comparisons were carried out between T2DM patients without DN and with either CIC- or BP-defined nephropathy. (a) No significant difference in genotype distribution was observed between T2DM patients with DN and without DN when applying CIC. The frequency of patients homozygous for the $(CTG)_5$ allele decreased when BP-DN was considered. However, this difference did not hold after Bonferroni correction. ((b) and (c)) Gender stratification ((b) male patients, (c) female patients) showed no significant difference in the frequency of homozygosity for the *CNDP1* $(CTG)_5$ allele between T2DM with and without DN when applying CIC. When DN was confirmed through biopsy, however, the frequency of *CNDP1* $(CTG)_5$ homozygosity significantly decreased in female T2DM patients, which remained significant after Bonferroni adjustment.

believed to be necessary for obtaining sufficient power (i.e., $n = 10$/independent variable). In the multivariate model, systolic blood pressure (OR = 1.024, $P = 0.045$) was positively and age (OR = 0.931, $P = 0.007$) and diabetes duration (OR = 0.895, $P = 0.025$) were negatively associated with biopsy-proven nephropathy, respectively.

3.3. CNDP1 Genotype Distribution over Time on Hemodialysis and Diabetes Duration. The *CNDP1* genotype distribution (*CNDP1* $(CTG)_5$ homozygous—versus all other *CNDP1* genotypes) was tested in 175 patients on hemodialysis, including 90 patients with DN according to CIC and/or BP-DN and 85 CIC-NDRD patients. Patients were stratified

Table 2: Summary of logistic regression analysis of variables predicting biopsy-proven diabetic nephropathy (male and female, $n = 160$).

	Univariate analysis			Multivariate analysis		
	OR	95% CI	P value	OR	95% CI	P value
Age (years)	0.920	0.881–0.961	<0.001	0.931	0.883–0.980	0.007
BMI (kg/m^2)	0.980	0.922–1.041	0.513	—	—	—
Diabetes duration (years)	0.886	0.813–0.966	0.006	0.895	0.812–0.986	0.025
HbA1c (%)	1.308	1.020–1.678	0.034	1.169	0.844–1.619	0.348
Male sex	2.333	0.994–5.477	0.052	1.957	0.748–5.124	0.171
SBP (mmHg)	1.021	1.000–1.042	0.052	1.024	1.001–1.047	0.045
CTG_5 homozygosity	0.353	0.127–0.984	0.047	0.307	0.096–0.980	0.046

Area under the ROC curve (AUC) = 0.797, $P = 0.862$ for Hosmer-Lemeshow test. BMI: body mass index; SBP: systolic blood pressure.

on the basis of hemodialysis duration, that is, time on dialysis: <30 months ($n = 60$), between 30 and 100 months ($n = 76$) and >100 months ($n = 39$). To assess the frequencies over time on hemodialysis and diabetes duration, the χ^2 test for trend was carried out.

The frequency of the $(CTG)_5$ homozygous genotype significantly increased with time on hemodialysis (<30 months: 33%, 30–100 months: 40%, >100 months: 49%, $P < 0.05$) (Figure 3(a)), while gender distribution was approximately equal in the groups. The association of the $(CTG)_5$ homozygous genotype with time on hemodialysis was not significant in the subgroup analyses after gender stratification (contingency tables not shown).

Although the frequency of the homozygous *CNDP1* $(CTG)_5$ genotype uniformly increased with diabetes duration, this trend did not reach statistical significance (<10 years: 27%, 10–15 years: 34%, 16–20 years: 37%, >20 years: 42%, $P = 0.17$) (Figure 3(b)). However, if patients with a heterozygous *CNDP1* $(CTG)_5$ genotype were included (i.e., patients with at least one $(CTG)_5$ allele), there was a clear, significant trend towards high frequencies with increasing diabetes duration (<10 years: 64%, 10–15 years: 75%, 16–20 years: 87%, >20 years: 90%, $P < 0.01$) (Figure 3(c)).

3.4. CN-1 Activities in Hemodialysis Patients. Because serum carnosinase (CN-1) activity correlates with *CNDP1* $(CTG)_n$ genotypes, that is, CN-1 activity is in general lower in individuals with less CTG copies, we also cross-sectionally assessed if serum CN-1 activity changes with time on dialysis. In line with the increased frequency of the homozygous *CNDP1* $(CTG)_5$ genotype in the groups of patients with a long history of hemodialysis, a significant negative correlation between serum CN-1 activity and log-transformed hemodialysis duration was found in all patients ($r = -0.33$; $P < 0.0001$, Figure 4(a)), T2DM patients only ($r = -0.034$, $P = 0.0006$, Figure 4(b)), and nondiabetic hemodialysis patients ($r = -0,031$, $P = 0.004$, Figure 4(c)).

To delineate if the *CNDP1* genotype is relevant for CN-1 activities in hemodialysis patients, patients on hemodialysis were stratified on the basis of homozygosity for the $(CTG)_5$ allele. Out of 174 subjects, the 65 patients carrying the homozygous *CNDP1* $(CTG)_5$ genotype showed a significantly lower serum CN-1 activity compared to patients with other genotypes (Figure 5(a), $P < 0.01$). This remained significant in female (Figure 5(c), $P < 0.05$), but not in male (Figure 5(b), $P = 0.07$) patients after gender stratification.

4. Discussion

This study examined whether the protection against DN afforded by the homozygous *CNDP1* $(CTG)_5$ genotype is still observed when applying clinical inclusion criteria or biopsy findings only and if the prevalence of the protective genotype changes in situations of increased cardiovascular mortality. Our results demonstrate that the frequency of the homozygous *CNDP1* $(CTG)_5$ genotype in the group of patients with biopsy-proven DN is significantly lower as compared to the groups of patients with no DN or with other biopsy-proven nephropathies. Our study also indicates that the frequency of homozygous *CNDP1* $(CTG)_5$ genotype tends to be higher in patients with a longer duration of hemodialysis, particularly in female patients. An analogous increase was detected for patients carrying at least one $(CTG)_5$ allele stratified for diabetes duration.

CIC for group allocations in DN studies bears the risk of wrongly assigning patients to the DN group as up to 20–50% of diabetic patients with albuminuria develop NDRD without concurrent DN [25, 26]. Although the presence of diabetic retinopathy (DR) is helpful for the prediction of DN [27] and thus improves the validity of group allocation, still DR may be absent in up to 50% of DN patients [28, 29]. Controversial studies reporting on genetic susceptibility loci for DN, including those for the *CNDP1* $(CTG)_n$ polymorphism, might partly underlie this problem.

The use of large cohorts from different consortia and subsequent meta-analysis of data obtained from genome-wide association studies (GWAS) may partly overcome this problem as the proportion of wrongly allocated patients might be outnumbered by the large number of studied patients. The GWAS approach has been successfully utilized in newer studies confirming susceptibility loci for declining glomerular filtration rate (eGFR) or albuminuria [30–32]. The *CNDP1* locus, a postulated DN susceptibility locus found by positional cloning [5] and case control studies [10], has

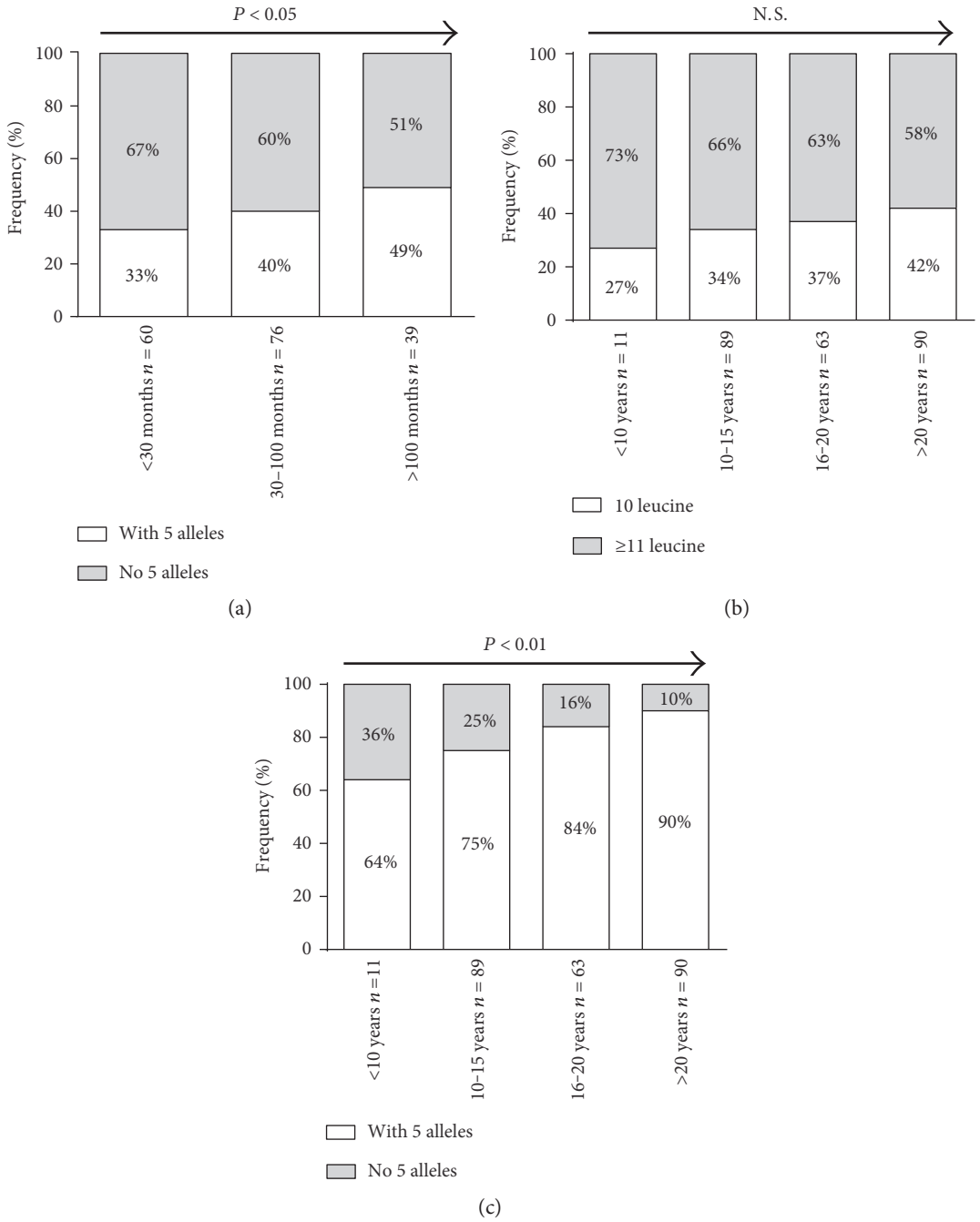

Figure 3: The *CNDP1* $(CTG)_5$ genotype distribution is changed with time on dialysis and diabetes duration. To assess if the frequencies change over time, the χ^2 test for trend (Cochran-Armitage test for trend) was carried out. (a) The frequency of the homozygous *CNDP1* $(CTG)_5$ genotype (10 leucine) significantly increased with time on hemodialysis. ((b) and (c)) Although the observed frequency of the homozygous (b) *CNDP1* $(CTG)_5$ genotype uniformly increased with diabetes duration, this trend did not reach statistical significance. Yet, if patients with a heterozygous (c) *CNDP1* $(CTG)_5$ genotype (one 5 allele) were included as well, a significant trend towards high frequencies with increasing diabetes duration was found. N.S.: not significant.

never been reported to be linked to DN in a GWAS approach, despite the fact that other genetic studies [11, 14, 33, 34] including a meta-analysis on 4546 DN, 7994 diabetes mellitus (DM), and 1826 healthy subjects [35] have confirmed an association between the *CNDP1* $(CTG)_n$ polymorphism and DN in T2DM patients. Significance further increased in these studies if more stringent CIC for DN, for example, the presence of proliferative DR and a longer duration of T2DM, were considered [11].

In our study, the association between the protective homozygous *CNDP1* $(CTG)_5$ genotype and DN was restricted to biopsy-proven DN and only significant in female patients using the χ^2 test. Multivariate logistic regression subsequently identified $(CTG)_5$ homozygosity as an independent protective

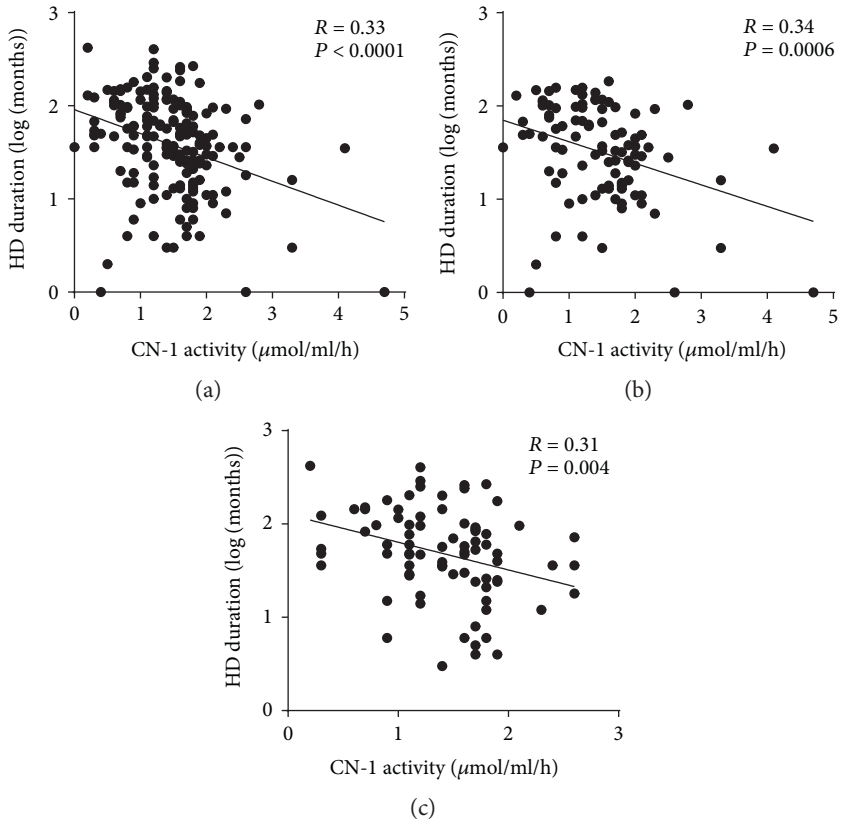

Figure 4: CN-1 activities decrease with time on dialysis. (a) Serum CN-1 activity was assessed in 175 hemodialysis patients and plotted against the log-transformed duration since hemodialysis was initiated. A significant correlation between serum CN-1 activity and log-transformed hemodialysis duration was found in all patients. ((b) and (c)) After stratification in T2DM (b) and other causes of renal failure (c), the correlation remained significant. HD: hemodialysis.

factor for biopsy-proven DN with an odds ratio of approximately 0.3. The negative association of age and diabetes duration with biopsy-proven DN in this analysis may be explained by the fact that in older diabetic patients, a renal biopsy is often waived due to the lack of consequence.

The frequencies of the homozygous *CNDP1* $(CTG)_5$ genotype in the no-DN and BP-NDRD groups were comparable, suggesting that this genotype does not afford protection against NDRD. Nonetheless, it would be prudent to be cautious with this assumption as other studies have suggested that this genotype also affords protection against other chronic kidney diseases (CKD), for example, glomerulonephritis but not tubulointerstitial nephritis [36]. In this light, the paradox between CIC-DN and BP-DN might be due to the fact that patients with NDRD were falsely included in the CIC-DN group, underscoring that clinical criteria do not provide a sufficient certainty for the diagnosis of DN in T2DM patients. It is important to note that patients in the BP-DN group had a shorter DM duration and displayed more severe hyperglycemia and albuminuria as compared to the CIC-DN group. Because of this relatively atypical DN course, these patients required a biopsy to clarify the actual underlying renal disease. Whether the change in *CNDP1* genotype distribution between CIC-DN and BP-DN underlies the severity of disease per se is unknown so far and cannot be excluded.

Our data are in agreement with a previous publication showing that the association of *CNDP1* and DN is most likely sex-specific [14]. Although also in males of the BP-DN group the frequency of the $(CTG)_5$ homozygous genotype was lower as compared to the no-DN group, it was only significantly decreased in female BP-DN patients. The sex-specific protection of *CNDP1* is generally explained by higher serum CN-1 activities in females [14].

In keeping with the recently published prospective study that the *CNDP1* genotype may impart a cardiovascular mortality risk in female, but not in male T2DM patients [19], we investigated whether genotype distribution changes with time on dialysis or diabetes duration. Since both of the latter variables are associated with an increased (cardiovascular) mortality risk, it would be expected based on the above study that the frequency of the homozygous *CNDP1* $(CTG)_5$ genotype would decrease rather than increase in patients with a long history of hemodialysis. By contrast, our data show that the frequency of the homozygous *CNDP1* $(CTG)_5$ genotype was significantly increased in patients with a long history of hemodialysis. This difference remained in both males and females although it was not statistically significant, which is likely explained by the small sample size of the subgroup analysis. Similar findings were also observed with respect to a longstanding diabetes duration when all patients carrying at least one $(CTG)_5$ allele were included.

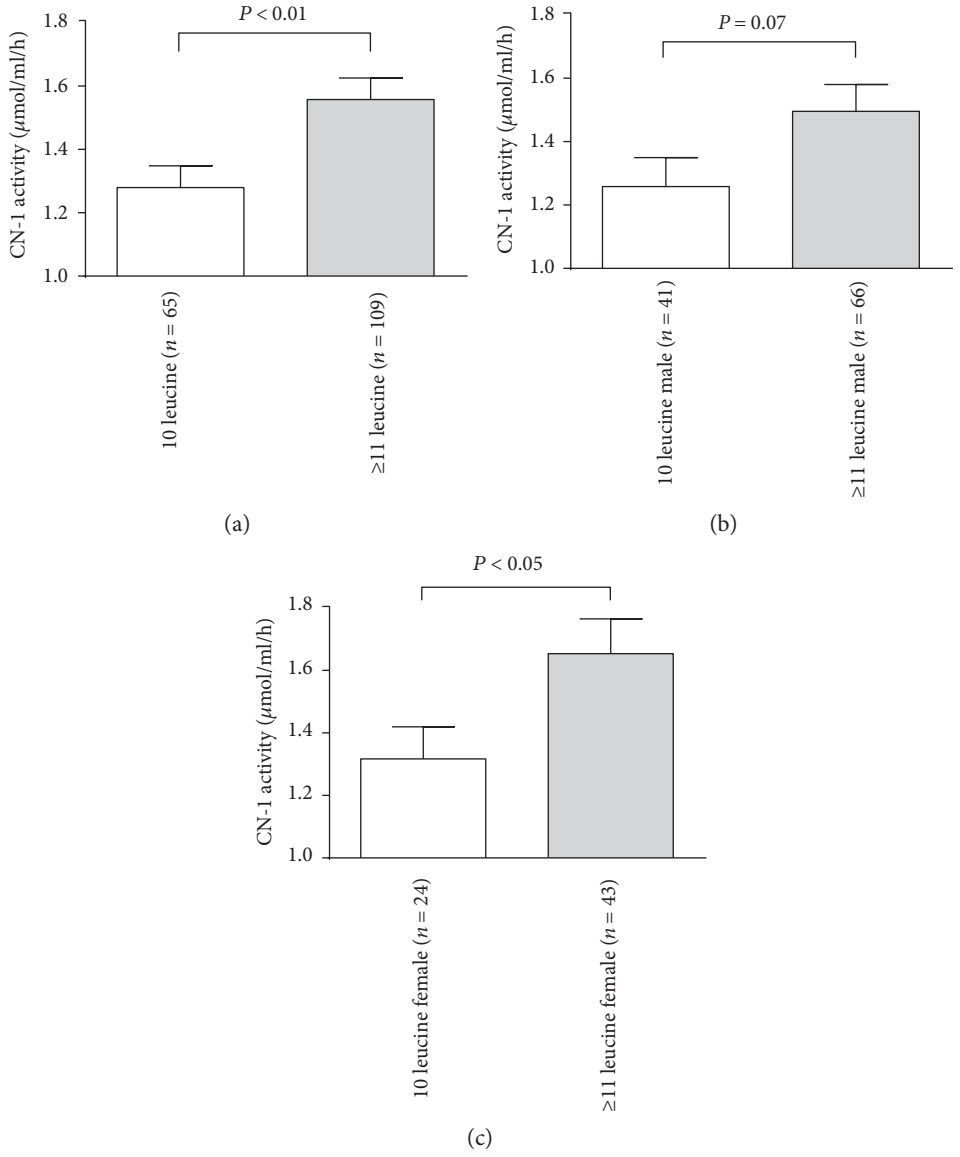

Figure 5: CN-1 activity correlates with *CNDP1* genotype in hemodialysis patients. (a) CN-1 activity in $(CTG)_5$ homozygous hemodialysis patients is significantly lower than that in patients carrying other genotypes. ((b) and (c)) After gender stratification differences in CN-1 activity between the $(CTG)_5$ homozygous, all other genotypes remained but only reached statistical significance in females (c).

Although our findings suggest that in patients on hemodialysis and in diabetic patients the *CNDP1* $(CTG)_5$ genotype may not impart an additional mortality risk, it should be underscored that the small group sizes and the cross-sectional design of this study impede drawing firm conclusions, in particular since a considerable number of hemodialysis patients were not diabetic. Nonetheless, these findings suggest that in patients on hemodialysis and in diabetes, the *CNDP1* $(CTG)_5$ genotype may not impart an additional mortality risk.

Serum CN-1 concentrations and activities are in part determined by $(CTG)_n$ polymorphism [10, 37]. Since this repeat is located in the hydrophobic part of the CN-1 signal peptide and is essential for the translocation of CN-1 protein during secretion, it is believed that the shorter $(CTG)_5$ variant is less efficiently secreted [37]. In line with an increased frequency of the homozygous *CNDP1* $(CTG)_5$ genotype in patients with a long history of hemodialysis, CN-1 activities were reduced. This reduction is not due to the loss of protein through hemofiltration since serum CN-1 concentrations even increase proportionally to the amount of ultrafiltrate [38].

As discussed above, we acknowledge the relatively small sample size as a major limitation of our study, accounting for a limited statistical power regarding major questions addressed. In addition, in contrast to a prospective study design, no systematic biopsy strategy with uniform indications and assigned nephropathologists could be implemented. Other studies, however, demonstrated that the histological classification of DN based on glomerulopathy shows a satisfying interobserver reproducibility [39]. We also acknowledge uncertainties regarding the

procedure of patient allocation leading to limited group selectivity. This holds true especially for the diabetic control group without DN, which is based only on clinical criteria instead of a histological diagnosis. As 80% of these patients were on ACE inhibitor or AT_1-blocking drugs, albuminuria alone was not a reliable parameter. Despite the extension of the criteria by diabetes duration of >15 years and exclusion of patients which manifest retinopathy, accidental assignment of patients with DN to this group is not improbable.

In our eyes, the fact that this study still resulted in significant results in the light of these conceptual drawbacks supports a particular strong association of our findings. Our study supports the hypothesis that protection against DN is indeed afforded by the *CNDP1* $(CTG)_5$ genotype and that this association mainly applies to female T2DM patients. The restriction of this finding to the BP-DN group may be attributed to false allocation of patients with other proteinuric diseases to the CIC-DN group. In fact, 20–50% of diabetic patients with proteinuria display NDRD without concurrent DN [25, 26]. Our investigation also suggests that $(CTG)_5$ homozygous hemodialysis patients and patients with diabetes carrying at least one $(CTG)_5$ allele might have a survival benefit as compared to other genotypes. These findings warrant further conformational studies, ideally with a prospective longitudinal design.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Thomas Albrecht and Shiqi Zhang are equally contributing authors.

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Review Article

Podocyte Autophagy: A Potential Therapeutic Target to Prevent the Progression of Diabetic Nephropathy

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Diabetic nephropathy (DN), a leading cause of end-stage renal disease (ESRD), becomes a worldwide problem. Ultrastructural changes of the glomerular filtration barrier, especially the pathological changes of podocytes, lead to proteinuria in patients with diabetes. Podocytes are major components of glomerular filtration barrier, lining outside of the glomerular basement membrane (GBM) to maintain the permeability of the GBM. Autophagy is a high conserved cellular process in lysosomes including impaired protein, cell organelles, and other contents in the cytoplasm. Recent studies suggest that activation of autophagy in podocytes may be a potential therapy to prevent the progression of DN. Here, we review the mechanisms of autophagy in podocytes and discuss the current studies about alleviating proteinuria via activating podocyte autophagy.

1. Introduction

Diabetes mellitus (DM) has been one of the global health issues. According to the report from the International Diabetes Federation, the number of patients with DM will increase to 205 million in 2035 than in 2014. Diabetic nephropathy (DN), a serious chronic complication of DM, is a leading cause of end-stage renal disease (ESRD). One significant clinical feature of DN is the appearance of urinary protein, defined as "albuminuria." Structural changes of the glomerular filtration barrier are detected in the diabetic patients with albuminuria, including glomerular endothelial cell injury, the loss of podocytes, glomerular basement membrane (GBM) thickening, and mesangial expansion [1, 2]. Apart from GBM dysfunction, the accumulation of advanced glycation end products (AGEs), oxidative stress, and the activation of the renin-angiotensin system (RAS) also contribute to the decline in renal function [1, 3–7].

Based on the pathologic alterations in the kidney, DN is classified into four groups: class I includes GBM thickening, class II consists of mild (IIA) to severe (IIB) mesangial expansion, class III includes nodular glomerulosclerosis, and class IV represents developed DN which is characterized with over 50% global glomerulosclerosis and podocyte deficiency [8, 9]. Among these four categories, the kidney may also exhibit arteriolar hyalinosis, glomerular capillary subendothelial hyaline, and arteriosclerosis. Present therapies are mainly focusing on the way to reduce the levels of blood glucose and blood pressure to normal, and most of them alleviate albuminuria via suppressing the RAS activity [10]. Nevertheless, considering the elevation of diabetic kidney diseases, further studies in a pathogenetic mechanism for DN are needed to find new approaches to treat DN. Recently, a number of reports have demonstrated that autophagy is involved in the pathogenesis of diabetes-related podocyte injury. In this review, we will make a summary on the role of autophagy in this process and the mechanisms involved.

2. Autophagy

Autophagy (from the ancient Greek meaning "self-eating") is a highly conserved cellular process that delivers protein and other impaired cell organelles to lysosomes for degradation and recycle to maintain intracellular homeostasis. Christian de Duve first referred autophagy in 1963 [11]. Subsequent studies focused on the regulatory mechanisms of autophagy and its effects on human health and disease.

On the basis of different ways of transporting intracellular constituents to lysosomes, autophagy is divided into three types: macroautophagy, microautophagy, and chaperone-mediated autophagy [12]. Macroautophagy and chaperone-mediated autophagy are through autophagosomes and chaperone complex, respectively, while constituents are delivered to lysosomes directly in microautophagy [13, 14]. In this review, macroautophagy (hereafter referred to as autophagy) is mainly investigated among these three types. In terms of different types of degraded substrates, autophagy was also divided into selective and nonselective autophagy. Degradation of some impaired organelles, lipophagy, or xenophagy is involved in selective autophagy, whereas deficient nutrient-induced autophagy is considered the nonselective type [15–17].

Autophagy, first detected in the yeast, is a complex process comprising of autophagy-related gene (Atg) product cooperation. Atg proteins are classified into five groups: Atg1 kinase complex [Atg1/Unc-51-like kinase (ULK) 1/2], Atg9, class III phosphoinositide 3-kinase complex (PI3KC3), and two ubiquitin-like conjugation systems (Atg12-Atg5 and Atg8 conjugation system) [18]. Besides the Atg regulation, there are some other regulatory mechanisms of autophagy, such as the mammalian target of rapamycin signaling pathway and cellular stress pathway [19–21].

3. Podocyte Autophagy in Diabetic Nephropathy

Studies have demonstrated that autophagy is renoprotective in acute kidney injury, obstructive nephropathy, diabetic nephropathy, and other renal diseases [22]. Podocytes are highly differentiated epithelial cells lining the outer aspect of the GBM with interdigitating foot processes, and the slit diaphragms between foot processes play a role in substance filtration. Podocyte injury including foot process fusion and slit diaphragm alteration results in abnormal permeability of the GBM, terminally leading to albuminuria. Autophagy controls the quality of the cytoplasm, via degrading proteins, peroxidases, and damaged organelles that complicate the recycle of organelles, and then maintains the homeostasis of intracellular environment [23, 24]. The self-repaired feature of autophagy is important in the anaphase cells such as neurocytes and podocytes, which have a restricted capacity in differentiation and proliferation [25]. The previous studies explored the mechanisms of podocyte autophagy in DN and suggested that activated podocyte autophagy has an effect on DN through an Atg12-Atg5 conjugation system, mTOR, adenosine 5′-monophosphate- (AMP-) activated protein kinase (AMPK), and oxidative stress as well as vascular endothelial growth factor.

3.1. Atg12-Atg5 Conjugation System in Podocyte Autophagy and Diabetic Nephropathy. Atg12 is a ubiquitin-like protein involving in autophagosome formation. Autophagy activation needs the conjugation of Atg12 to Atg5, which is stimulated by Atg7 and Atg10, and then promotes Atg8 and lipid phosphatidylethanolamine conjugation in the cytoplasm [26]. The activation of the Atg12-Atg5 conjugation system promotes the production of autophagosome and then activates podocyte autophagy. Currently, Liu et al. demonstrated that β-arrestins, a negative adaptor of G protein-coupled receptors (GPCRs), aggravate podocyte injury through autophagy inhibition in DN [27]. They found that β-arrestins suppressed podocyte autophagy via downregulating Atg12-Atg5 conjugation, which is induced by enhancing the interaction between β-arrestins and Atg7. Therefore, modulation of this pathway may be a novel therapeutic approach for treating patients with DN.

3.2. mTOR Signaling Pathway in Podocyte Autophagy and Diabetic Nephropathy. Mammalian target of rapamycin (mTOR) is essential to cell growth regulation, and activation of mTOR suppresses autophagy. Deficient nutrients (such as growth factor or amino acid deficiency) in the cytoplasm activate autophagy by suppressing the expression of mTOR. After inhibition, mTOR not only can activate the formation of class III phosphatidylinositol 3-kinase (PI3K) complex and the unc-51-like kinase (Ulk) 1 complex but also inhibit the activity of ribosome protein subunit 6 kinase 1 (S6K1) [28–31]. In the upstream of mTOR, there are two separated protein kinases, phosphatidylinositol 3-kinase I (PI3K-I)/protein kinase B and AMP-activated protein kinase, which are regulated by different conditions [32].

3.2.1. Phosphatidylinositol 3-Kinase I (PI3K-I)/Protein Kinase B (Akt/PKB). PI3Ks are consisted of three isoforms, including class I, class II, and class III [33, 34]. As a member of Atg proteins, class III PI3K composes of a Vps15 regulatory subunit and a Vps34 catalytic subunit, which promote phosphatidylinositol (PI) conversion to phosphatidylinositol 3-phosphate [PI(3)P] and then initiate autophagy [35–38]. In contrast, the class I PI3K regulatory subunit p58 is bonded to the catalytic subunit p110 and then activates the Akt/mTOR signaling pathway [39, 40] by promoting phosphatidylinositol 3,4,5-triphosphate. Therefore, it seems that class I PI3K inhibits autophagy while class III PI3K activates it. The activation of class I PI3K is triggered by insulin or growth factors to interact with insulin receptors or tyrosine kinase receptors, which are the members of transmembrane receptors existing on the membrane of podocytes and then activates Akt/PKB. Then, the downstream tuberous sclerosis complex 1 and 2 (TSC1/2) proteins will be inhibited by PKD1 and the production of Akt/PKB activation. In the end, podocyte autophagy is suppressed by the activation of mTOR.

Recent studies have emphasized the relationship between DN and nutrient-dependent pathways, involving the mTOR signaling pathway. In the models of diabetic nephropathy, especially the type 1, insulin resistance blocks the phosphorylation of Akt/PKB and then activates mTOR by increasing the expression of Rheb (Ras homolog enriched in brain). Thus, insulin resistance suppresses podocyte autophagy through increasing the activity of mTOR.

3.2.2. AMP-Activated Protein Kinase (AMPK). As an essential regulator in energy metabolism, AMPK is an enzyme consisted of three proteins (α, β, and γ) [41]. AMPK can be activated by an increase in Ca^{2+} concentration in the

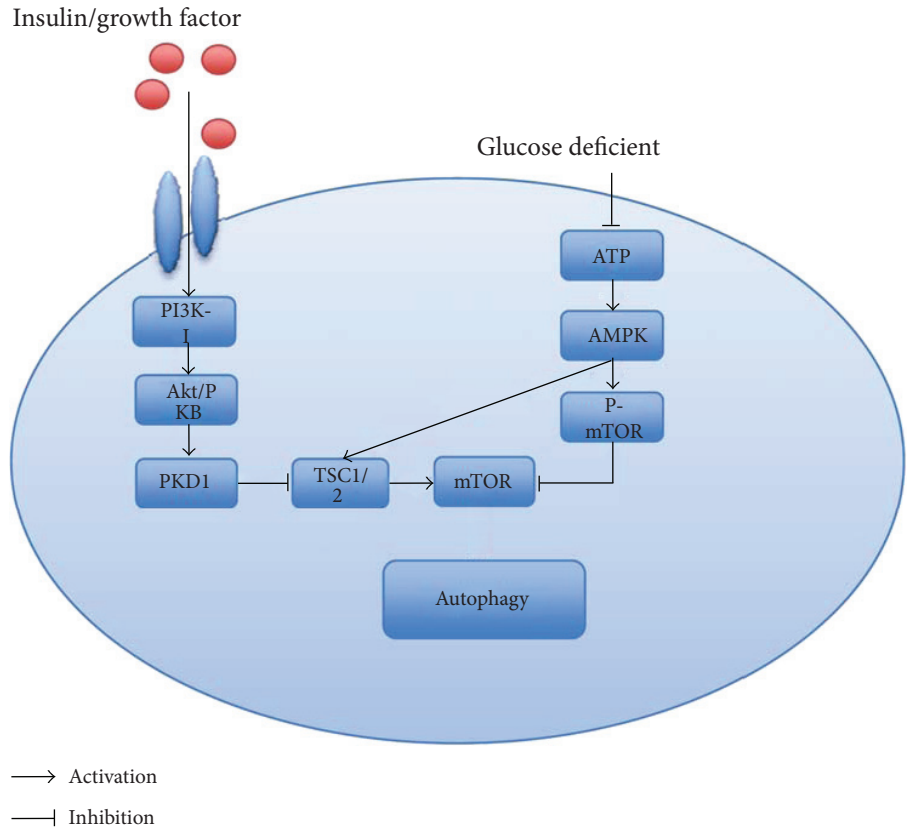

Figure 1: mTOR signaling pathway in podocyte autophagy. PI3K-I: class I phosphatidylinositol 3-kinase; Akt/PKB: protein kinase B; TSC: tuberous sclerosis complex; ATP: adenosine triphosphate; AMPK: AMP-activated protein kinase.

cytoplasm [42, 43] and the stimulation of numerous hormones, adipokines, and cytokines. In addition to these, the ratio of an intracellular AMP/ATP decrease also activates AMPK. Nutrient starvation induced the activation of AMPK. In the condition of ATP deficiency, the downstream TSC1/2 is activated by AMPK, then inhibits Rheb, and finally enhances autophagy by suppressing mTOR activation (Figure 1). Recently, Jin et al. suggested that berberine alleviated high glucose-induced apoptosis of podocytes in mouse via increasing the activity of AMPK [44]. They showed that the expression of p-AMPK in a high-glucose (HG) group was lower and the expression of p-mTOR was higher in the HG group compared with the control group, while these results were conversed by berberine administration.

Mechanical stress induced by the renin-angiotensin system is considered a major damage factor in podocytes of DN. Spironolactone, a common diuretic, is generally used to treat heart failure, edema, or Conn's syndrome. The study from Li et al. demonstrated that spironolactone has renoprotective effects on activating autophagy through blockage of the mTOR signalling pathway in podocytes under mechanical stress [45]. They used the Flexercell FX-5000TM Compression System to establish the animal model of DN and found that the expressions of p85-PI3K, p-AKT, and p-mTOR were significantly increased compared with those of the control group. After administration of spironolactone for 48 h, the levels of p85-PI3K, p-AKT, and p-mTOR were markedly decreased, which are in accordance with the results in the group by PI3K inhibition. Thus, spironolactone might be a new therapy of DN.

Rapamycin is a new immunosuppressive drug of macrocyclic lactone, which was first found in a soil bacterium in 1965 [46]. After that, researchers suggested that rapamycin has antifungal effects as well as anti-T cell activity in succession [47]. Furthermore, rapamycin is a selective inhibitor of mTOR [48]. Rapamycin binding to immunophilins, such as FKBP12 (*FK* binding protein, 12 kDa), forms an FKBP12-rapamycin complex. The FKBP12-rapamycin complex suppresses the expression of mTOR through phosphorylation of mTOR and then activates autophagy. However, the number of clinical trials of rapamycin in DN is less; further studies are needed to clarify the renoprotective property of rapamycin in DN.

3.3. Reactive Oxygen Species (ROS) in Podocyte Autophagy and Diabetic Nephropathy. Besides insulin and nutrition starvation, intracellular metabolism alternations are also related to the pathogenesis of DN, involving the increase in reactive oxygen species (ROS). Several studies have shown that ROS are the most common factors in activating podocyte autophagy. An increase in ROS production activates PKR-like kinase (PERK), which then oxidizes Atg4 proteases via eIF2a phosphorylation, subsequently promotes the level of proteolytic mature LC3, and prevents mTOR activation [49] (Figure 2). Recently, Ma et al. explored the effect of high-glucose milieu on podocyte autophagy and suggested

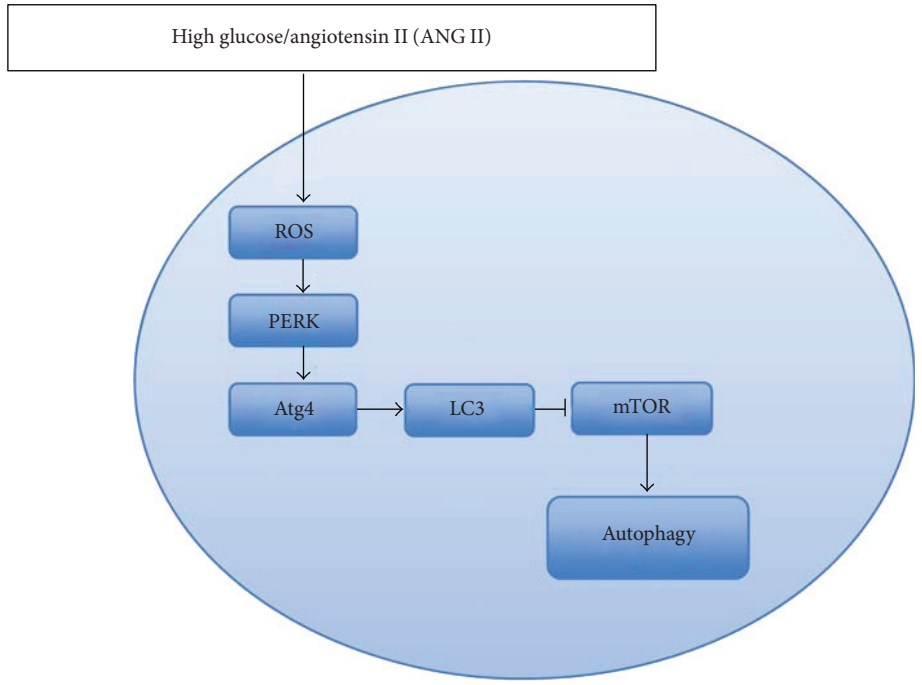

Figure 2: Reactive oxygen species (ROS) in podocyte autophagy. ROS: reactive oxygen species; PERK: PKR-like endoplasmic reticulum kinase.

that podocyte autophagy was activated by upregulating the generation of mitochondrial ROS after exposing to high glucose for 24 hours [50]. Meanwhile, podocytes exposed to angiotensin II (ANGII) also increased the generation of ROS and promoted autophagy activation [51]. However, the membrane of the mitochondrion is damaged by excessive ROS generation in the mitochondrion, and ROS releasing into the cytoplasm may cause damage to other organelles. Since the function of autophagy targeting and degrading injury organelles is selective, the augmentation of ROS will be limited [52]. Chronic exposure to high-glucose condition leads to autophagy insufficiency and subsequently causes lysosomal dysfunction and podocyte apoptosis, finally resulting in diabetic nephropathy [53]. Therefore, reduction of ROS generation is a potential therapeutic approach for preventing the development of DN.

3.4. Vascular Endothelial Growth Factor (VEGF) in Podocyte Autophagy and Diabetic Nephropathy. In the early phases of animals or patients with DN, the level of VEGF has been shown to be increased in the kidney. Several studies have suggested that elevation of VEGF is associated with the increase in the glomerular permeability, then resulting in proteinuria [54].VEGF is considered to be a promoter of angiogenesis and synthesized mainly by the podocytes. VEGF-A, as one member of a VEGF family, has a negative effect on glomerular endothelial cell (GEC) glycocalyx through the early stages of DN, and this effect can be reversed by VEGF-A_{165b}, an inhibitory isoform of VEGF-A, finally ameliorating proteinuria [55]. Autophagy has been reported to prevent angiogenesis [56, 57]. Miaomiao et al. found that high glucose enhanced the level of VEGF, whereas this elevation is downregulated by autophagy activation via rapamycin, an inhibitor of mTOR [58]. Yang [59] and Liu et al. [60] also demonstrated that the increase in autophagosome inhibits angiogenesis.

4. Conclusion

According to the International Diabetes Federation, the global diabetes prevalence will increase from 8.3% in 2014 to 10.1% in 2053. As a serious global health issue, it is urgent to find potent therapies to treat diabetes and its complications, especially diabetic nephropathy. The previous studies have shown the activation of autophagy in podocytes via inhibiting the expression of mTOR and alleviating albuminuria in DN. Meanwhile, autophagy activation also decreased the expression of VEGF and subsequently prevented the progression of DN. Although studies have suggested that podocyte autophagy is a renoprotective process in lysosome, DN is an extremely complex complication. Further investigations are needed to elucidate the role of autophagy in podocyte injury induced by DN and discover the autophagy-based therapies for the treatment of DN.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Na Liu and Liuqing Xu are co-first authors.

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Research Article

The Association of a Genetic Variant in *SCAF8-CNKSR3* with Diabetic Kidney Disease and Diabetic Retinopathy in a Chinese Population

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Background. Genome-wide association studies found rs955333 located in 6q25.2 was associated with diabetic kidney disease in multiple ethnic populations, including European Americans, African Americans, and Mexican Americans. We aimed to investigate the association between the variant rs955333 in *SCAF8-CNKSR3* and DKD susceptibility in Chinese type 2 diabetes patients. *Methods*. The variant rs955333 was genotyped in 1884 Chinese type 2 diabetes patients. Associations of the variant rs955333 with DKD and DR susceptibility and related quantitative traits were evaluated. *Results*. The variant rs955333 was not associated with DKD in our samples, while subjects with genotype GG were associated with DR ($P = 0.047$, OR = 0.5525 [0.308, 0.9911]), and it also showed association with microalbuminuria ($P = 0.024$, beta = −0.1812 [−0.339, −0.024]). *Conclusion*. Our data suggests the variant rs955333 was not associated with DKD but showed association with diabetic retinopathy in Chinese type 2 diabetes patients.

1. Introduction

Diabetic kidney disease is a leading cause of end-stage kidney disease (ESKD) globally and continues to grow over decades [1]. A recent study shows that chronic kidney disease accompanied diabetes has become more widespread than primary glomerulonephritis in China [2]. Intensification of glycemic and blood pressure and lipid control has not markedly reduced the incidence of DKD [3]. There might be any other factors which play an important role in occurrence and development of the disease [4]. The evidence including aggregation in families, variable prevalence rates between different race, and the highly heritable trait in clinic and histology indicates that genetic factors play an important role in the pathogenesis of DKD [5–8]. Genome-wide association studies have identified that several loci are associated with DKD in different population [9–14].

A genetic variant rs955333 within *SCAF8-CNKSR3* has been identified to be associated with DKD and reach genome-wide significance in multiple ethnic populations, including European Americans, African Americans, and Mexican Americans [15]. This Single Nucleotide Polymorphism (SNP) is located in a region between the SR-like carboxyl-terminal domain associated factor 8 gene *(SCAF8)* and the connector enhancer of KSR family of scaffold proteins gene *(CNKSR3)*, which may suggest that this SNP may regulate transcription of genes in the region [15]. *CNKSR3* is an aldosterone receptor target gene and it mainly regulates sodium absorption in the distal nephron to maintain plasma volume and blood pressure [16]. *SCAF8* is RNA maturation factor and plays a role in mRNA processing [15].

Although this SNP shows strong relationship with DKD in FIND's study [15], there is no data about its association with DKD in Chinese population and relevant quantitative

trait. Therefore, we performed the present study, aimed to test if rs955333 played a role in genetic susceptibility of DKD in Chinese population.

2. Methods

2.1. Ethical Approval. According to Helsinki Declaration II, ethical approval was granted by the institutional review board of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Oral and written informed consent were obtained from each participant.

2.2. Participants. In this study, we recruited 1884 unrelated Chinese Han subjects from the Shanghai Diabetes Institute Inpatient Database of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. All participants were T2DM patient who were eastern Chinese Han ancestry and meet the 1999 WHO criteria (fasting plasma glucose ≥ 7.0 mmol/L and/or 2 h plasma glucose ≥ 11.1 mmol/L). Type 1 diabetes and mitochondrial diabetes were excluded by clinical, immunological, and genetic criteria, and patients with an estimated glomerular filtration rate albuminuria ≥ 30 mg/24 h or (eGFR) < 90 mL/min per 1.73 m^2 or ACR (albumin-to-creatinine ratio) ≥ 30 μg/mg were diagnosed as DKD. Of these patients, 508 were diagnosed with diabetic kidney disease and 1376 had diabetes for longer than 5 years but without diabetic kidney disease and were selected as cases and controls, respectively, 545 were diagnosed with diabetic retinopathy, and 1120 subjects who had diabetes for longer than 5 years but without diabetic kidney disease were chosen as control.

2.3. Clinical Measurement. The history of every patient was recorded in detail, as well as the anthropometric and biochemical data. The patients' height (/m) and weight (/kg) were measured, and their body mass index (BMI) was calculated as weight/height^2. Blood pressure was also measured by standard measurements. HbA1c levels were surveyed by high performance liquid chromatography (HPLC) by a Bio-Rad Variant II Haemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA, USA). The albuminuria level was measured with scatter turbidimetry by BN II System (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany), the sample came from 24 h urine collection, the measurement was repeated in three different days, and the mean values of these measurements were used for further analysis. The eGFR was calculated by a modification of the diet in renal disease (MDRD) study equation specially designed for a Chinese population [17]. Fundus photography of all subjects was performed with a 45° 6.3 megapixel digital nonmydriatic camera (Canon CR6-45NM; Lake Success, NY, USA).

2.4. SNP Selection, Genotyping, and Quality Control. The SNP reported by Family Investigation of Nephropathy and Diabetes (FIND), which reaches genome-wide significance, were genotyped. Genotyping was performed by primer extension of multiplex products with detection by matrix-assisted laser desorption ionisation-time of flight mass spectroscopy by a MassARRAY Compact Analyzer (Sequenom, San Diego, CA, USA). Approximately, 1884 individuals and SNP rs955333 were reserved for further analysis after the quality control.

2.5. Statistical Analysis. The allelic frequencies were compared between patients with or without DKD using a χ^2 test in PLINK (v1.07; http://pngu.mgh.harvard.edu/~purcell/plink) [18] and ORs with 95% CIs are presented. Genotype distributions between patients with or without DKD were compared using logistic regression under additive and recessive models with adjustment of confounding factors. Quantitative traits related to diabetic microvascular disease were preformed using multiple linear regression under additive and recessive models with adjustment of confounding factors. The statistical analyses were performed using SAS 9.3 (SAS institute, Cary, NC, USA) unless specified otherwise. A two-tailed P value < 0.05 was considered statistically significant. On the basis of an estimated effect size of genetic loci for DKD (~0.73), our samples had >95% power to detect SNP with minor allele frequency of 0.2 at a level of significance of 0.05 by Quanto [19].

3. Results

The SNP rs955333 were successfully genotyped in 1884 individuals in the present study, without the basis of deviations from Hardy-Weinberg equilibrium ($P = 0.77$ in all subjects). We analyzed the association between the SNP and DKD and DR and related quantitative trait. The clinical characteristics of control and case of DKD or DR were shown in Tables 1 and 2, respectively. The results showed that there are significant differences between DKD cases and controls in gender, age, systolic blood pressure, diastolic blood pressure, AER, eGFR, uric acid, creatinine, blood urea nitrogen, total cholesterol, and total triglycerides ($P < 0.05$). Similarly, age, duration of diabetes, systolic blood pressure, and AER are also significantly different between DR cases and controls ($P < 0.05$).

The allele frequency of the variant showed no difference between DKD group and no-DKD group ($P = 0.8991$) (Figure 1(a)), while it showed some level of difference between diabetic retinopathy group and no-diabetic retinopathy group ($P = 0.053$) (Figure 1(b)).

Then we tested the association between the SNP and susceptibility of DKD under additive or recessive model, respectively. It showed that genotype GG was also lack of association with DKD under additive model ($P = 0.7003$) and recessive model ($P = 0.1109$, OR = 0.64 [0.3709, 1.108]) (Figure 1(c)). After that, we used logistic regression adjusting confounding factors including age, gender, BMI, duration, Hba1c, systolic blood pressure, diastolic blood pressure, total cholesterol, and total triglycerides to analyze the association between this rs955333 and DKD. Results suggested no association.

We also tested the association between the SNP and susceptibility of DR under additive or recessive model, respectively. It showed that rs955333 might be associated with DR both on the additive model ($P = 0.0546$, OR = 0.8342 [0.6934, 1.004]) and on the recessive model ($P = 0.0503$, OR = 0.5767 [0.3323, 1.001]) (Figure 1(d)). After adjusting confounding factors including age, gender, BMI, duration, Hba1c, systolic blood pressure, diastolic blood pressure, total

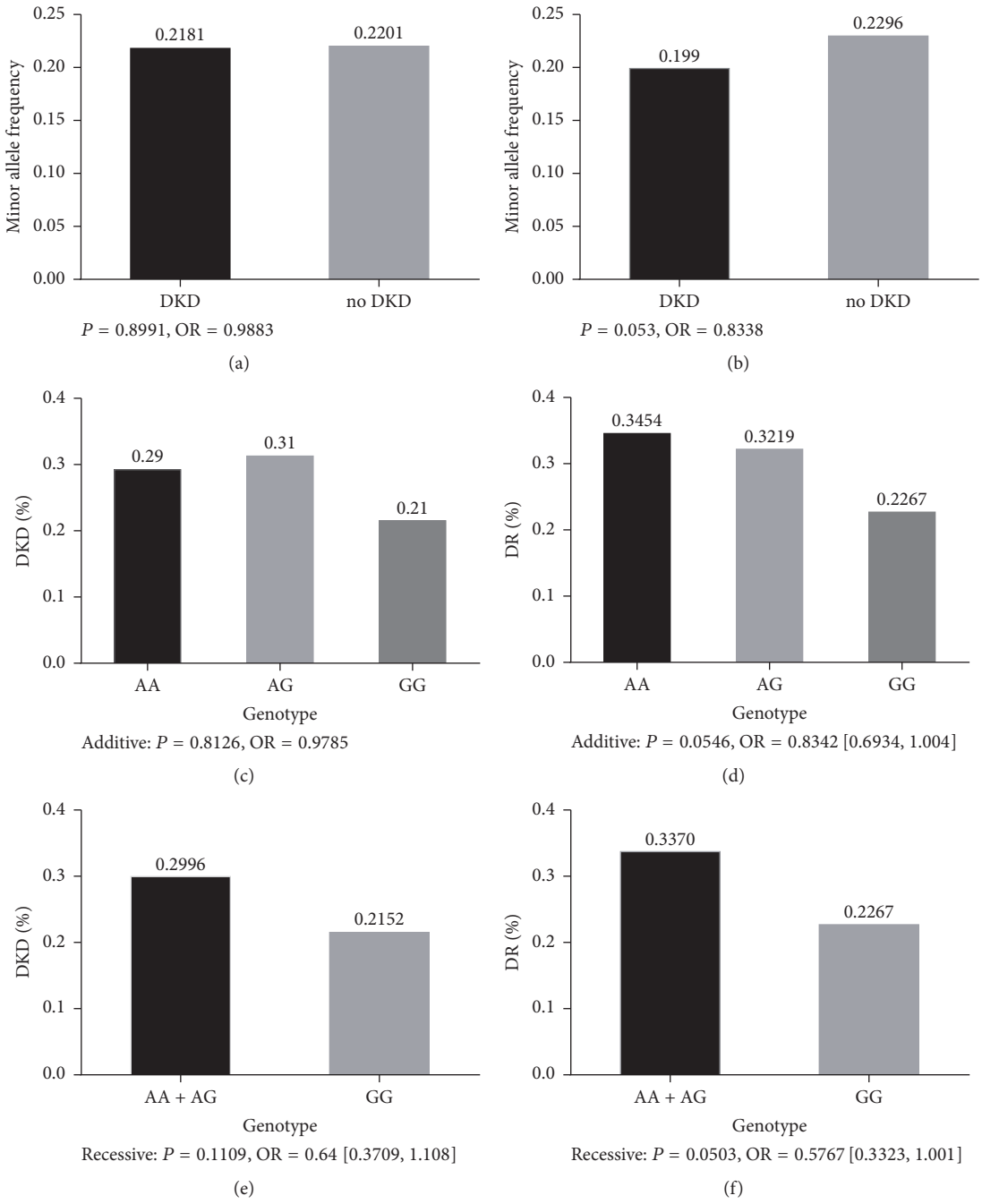

Figure 1: Allele frequency and prevalence rate in DKD and DR case-control study. (a) and (b) represent the association analysis in two case-control groups. The histograms represent the frequency of rs955333[G]. (c) and (d) represent the difference of prevalence rate in different genotypes on additive model. The histograms represent the prevalence rate. (e) and (f) represent the difference of prevalence rate in different genotypes on recessive model. The histograms represent the prevalence rate.

cholesterol, and total triglycerides, the result showed that subjects with genotype GG were associated with DR (P = 0.047, OR = 0.5525 [0.308, 0.9911]).

As for the association of the variant rs955333 with eGFR and 24-hour urinary albumin excretion rate (AER) by multiple linear regression analysis adjusting confounding factors including age, gender, BMI, duration, Hba1c, systolic blood pressure, diastolic blood pressure, total cholesterol, and total triglycerides, it showed association with AER (P = 0.024, beta = −0.1812 [−0.339, −0.024]) (Figure 2).

4. Discussion

Our study aimed to test the genetic association between SNP rs955333 and diabetic kidney disease in Chinese population. The DKD related SNP which was on human chromosome

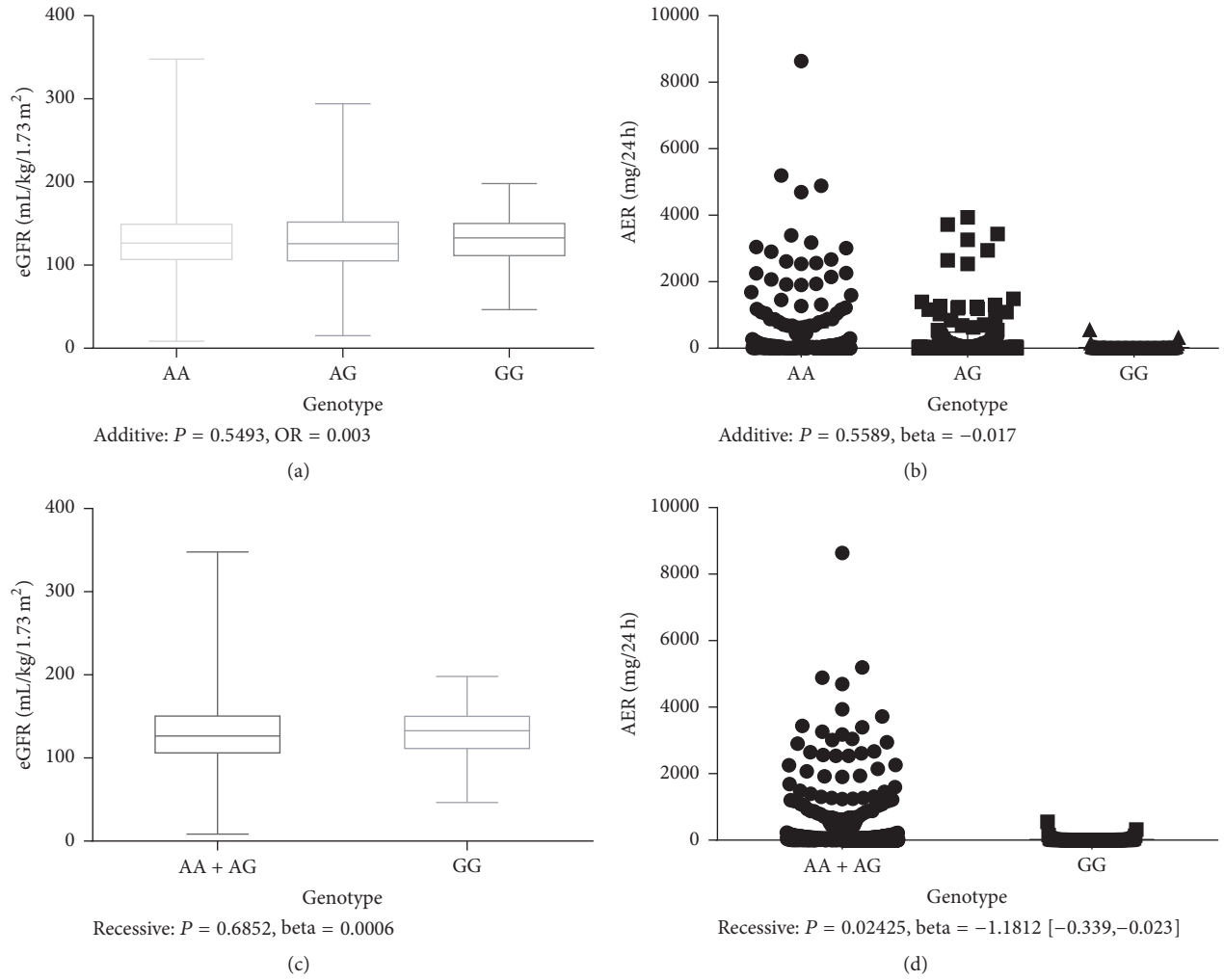

Figure 2: The difference of eGFR and AER in different genotypes. Box plot (a) shows the association of rs955333 with eGFR on additive model. Scatter plot (b) shows the association of rs955333 with AER on additive model. Box plot (c) shows the association of rs955333 with eGFR on recessive model. Scatter plot (d) shows the association of rs955333 with AER on recessive model. P values and beta values were determined by multiple linear regression adjusting for age, gender, BMI, duration, Hba1c, DP, SP, TC, and TG.

6q25.2 between the *SCAF8* and *CNKSR* genes was found by Family Investigation of Nephropathy and Diabetes (FIND) using genome-wide association studies in multiple population [15]. Although we performed the association study in 1884 Chinese Han, we could not find obvious evidence of association between rs955333 and DKD in our sample. However, we found that this SNP was marginally associated with DR in both additive and recessive model. After we adjusted the confounding factor, it showed the subjects with GG genotype could be protected from diabetic retinopathy (P = 0.047). Our results also showed that subjects with GG had lower level of microalbuminuria than other genotypes. So we concluded that rs955333 was associated with diabetic retinopathy. Diabetic retinopathy is a microvascular complication of diabetes, just like DKD. Although clinical data shows DKD patients are always accompanied with diabetic retinopathy, not all the patients with diabetic retinopathy will develop DKD at last [20]. It suggests that both shared and complication-specific mechanisms contribute to the different microvascular disease phenotypes. Our result shows that the subjects with genotype GG on rs955333 have lower prevalence rate of diabetic retinopathy and lower level of microalbuminuria. We speculated genotype GG on rs955333 might influence the expression level of the genes on 6q25.2. Like *CNKSR3* which is an aldosterone receptor target gene and highly expressed in the renal cortical collecting duct and is upregulated in response to physiologic aldosterone concentrations to keep plasma volume [16], further research needed to test whether genotype GG on rs955333 could lower the expression of *CNKSR3* so as to lower the prevalence of diabetic retinopathy.

In this study, we did not repeat the same results as FIND, one possible reason might be that the statistical power of our sample was not enough to detect the effect of this locus, and although we had over 90% power to detect the association at the level based on previously reported ORs in non-Asian population (0.73), we could not exclude the possibility that the SNP might be less effective in Chinese population than

Table 1: The clinical characteristics of DKD group and control group.

Characteristic	Control subjects	Diabetic kidney disease	*P* value
Male/female	719/570	326/209	**0.0427**
Age (years)	57.88 ± 9.81	60.14 ± 9.84	**0.0051**
BMI (kg/m^2)	24.82 ± 3.53	25.02 ± 3.49	0.3047
Duration of diabetes (years)	10 (7, 14)	10 (6, 15)	0.8527
HbA1C (%)	8.75 ± 2.07	8.85 ± 2.11	0.3727
SBP (mmHg)	131.04 ± 16.28	135.45 ± 17.36	<**0.0001**
DBP (mmHg)	79.92 ± 9.21	81.31 ± 9.61	**0.0127**
MA (mg/24h)	10.85 (6.41, 25.17)	58.13 (33.79, 157.16)	<**0.0001**
eGFR (mL/kg/1.73 m^2)	132.89 (115.95, 154.12)	101.59 (80.2, 132.25)	<**0.0001**
Uric acid (μmol/L)	301.0 (255.0, 359.0)	346.0 (287.0, 413.0)	<**0.0001**
Creatinine (μmol/L)	63 (53.37, 73.54)	80.12 (64.49, 101.29)	<**0.0001**
Blood urea nitrogen (mmol/L)	5.14 (4.23, 6.01)	6.04 (4.91, 7.53)	<**0.0001**
TC (mmol/L)	1.70 ± 1.4	2.11 ± 2.01	**0.0467**
TG (mmol/L)	4.70 ± 1.08	4.87 ± 1.37	<**0.0001**

Data are shown as the mean ± SD or median (interquartile range). The Wilcoxon test was used for the skewed distributed variables. χ^2 test was used to determine proportions of the categorical variables. *P* values < 0.05 are shown in bold. DKD: diabetic kidney disease. BMI: body mass index. SP: systolic blood pressure. DP: diastolic blood pressure. MA: microalbuminuria. eGFR: estimated glomerular filtration rate. TC: total cholesterol. TG: total triglycerides.

Table 2: The clinical characteristics of diabetic retinopathy group and control group.

Characteristic	Control subjects	Diabetic retinopathy	*P* value
Male/female	628/492	319/226	0.3414
Age (years)	58.56 ± 9.87	57.76 ± 9.24	**0.0473**
BMI (kg/m^2)	24.88 ± 3.53	24.84 ± 3.50	0.6125
Duration of diabetes (years)	9 (5, 12)	11 (8, 16)	<**0.0001**
HbA1C (%)	8.82 ± 2.1	8.80 ± 2.05	0.8048
SBP (mmHg)	130.67 ± 16.01	135.24 ± 17.68	<**0.0001**
DBP (mmHg)	80.35 ± 9.25	80.41 ± 9.29	0.8808
MA (mg/24h)	7.31 (3.46, 19.64)	8.34 (3.95, 26.33)	**0.0484**
eGFR (mL/kg/1.73 m^2)	126.08 (106.75, 148.52)	128.75 (105.72, 151.72)	0.7612
Uric acid (μmol/L)	309.5 (258.0, 369.0)	317.0 (269.0, 372)	0.1255
Creatinine (μmol/L)	65.5 (54.0, 78.0)	66.0 (55.0, 78.0)	0.4402
Blood urea nitrogen (mmol/L)	5.3 (4.4, 6.3)	5.3(4.4, 6.4)	0.8090
TC (mmol/L)	1.84 ± 1.72	1.80 ± 1.72	0.8687
TG (mmol/L)	4.77 ± 1.15	4.76 ± 1.21	0.4843

Data are shown as the mean ± SD or median (interquartile range). The Wilcoxon test was used for the skewed distributed variables. χ^2 test was used to determine proportions of the categorical variables. *P* values < 0.05 are shown in bold. DKD: diabetic kidney disease. BMI: body mass index. SP: systolic blood pressure. DP: diastolic blood pressure. MA: microalbuminuria. eGFR: estimated glomerular filtration rate. TC: total cholesterol. TG: total triglycerides.

other populations [21]. In this case, our sample may not have sufficient power. Secondly, the criteria we used to define DKD might be different from FIND, the subjects recruited by FIND (all had DM duration > 5 years and/or DR, with UACR > 1 g/g or ESKD) were more severe compared to us [15].

There are some limitations in our study. Firstly, all the subjects were located in shanghai or nearby regions; the population may have inherent bias. Second, subjects with advanced DKD should be recruited to further verify the genetic effect of the locus on DKD. Thirdly, the GWAS showed that the region 6q25.2 was associated with DKD, but we only tested one locus in our sample. On account of the population differences in the genetic architecture, we need to explore the region further.

5. Conclusion

Our data suggests rs955333 on chromosome 6q25.2 may not play a major role in DKD in Chinese population. However, we found evidence for association of rs955333 with

diabetic retinopathy susceptibility in Chinese people with T2DM.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Zhihong Liu, Weiping Jia, and Cheng Hu conceived of and designed the experiments. Li Jin, Tao Wang, and Rong Zhang performed the experiments. Li Jin, Tao Wang, and Cheng Hu analyzed the data. Li Jin, Miao Chen, Cheng Hu, Weiping Jia, and Zhihong Liu wrote the paper. Li Jin and Miao Chen drafted the manuscript. Li Jin, Tao Wang, Song Jiang, Miao Chen, Rong Zhang, Cheng Hu, Weiping Jia, and Zhihong Liu read and approved the final manuscript.

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Review Article

Epigenetic Regulations in Diabetic Nephropathy

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Diabetic nephropathy (DN) is a chronic complication of diabetes and the most common cause of end-stage kidney disease. It has been reported that multiple factors are involved in the pathogenesis of DN, while the molecular mechanisms that lead to DN are still not fully understood. Numerous risk factors for the development of diabetic nephropathy have been proposed, including ethnicity and inherited genetic differences. Recently, with the development of high-throughput technologies, there is emerging evidence that suggests the important role of epigenetic mechanisms in the pathogenesis of DN. Epigenetic regulations, including DNA methylation, noncoding RNAs, and histone modifications, play a pivotal role in DN pathogenesis by a second layer of gene regulation. All these findings can contribute to developing novel therapies for DN.

1. Introduction

Diabetic nephropathy (DN) is characterized by glomerular hypertrophy, proteinuria, decreased glomerular filtration, and renal fibrosis resulting in the loss of renal function. DN is a major complication associated with both type 1 and type 2 diabetes [1]. More than 1/2 of patients with type 2 diabetes and 1/3 of those with type 1 diabetes develop kidney disease, and DN is a prime reason for dialysis in many developed countries [2]. DN is classified as a small blood vessel complication of diabetes. Both ethnicity and inherited genetic differences are proposed for response to the development of DN [3, 4]. Intraglomerular hypertension and hyperfiltration are triggered because of the hyperglycemia, insulin resistance, and aberrant hemodynamics. However, the molecular mechanisms leading to DN are still not fully understood. Recently, there is emerging evidence that suggests the important role of epigenetic mechanisms in the pathogenesis of DN. It is rather remarkable that individuals who are exposed to hyperglycemia more likely develop diabetic complications. This phenomenon is referred to as metabolic memory or the legacy effect [5].

Epigenetics refers to heritable patterns of gene expression that are not dependent on the DNA sequence information. It has been studied in a variety of organisms [3]. The epigenetic modifications include cytosine methylation of DNA (DNA methylation), histone posttranslational modifications (PTMs), and noncoding RNAs [6]. The most well-known and best-characterized epigenetics are DNA methylation, which converts the DNA nucleotide, cytosine, into 5-methylcytosine. Another important epigenetic mark is the modification of histone proteins around which less than two turns of DNA are wound to form the nucleosome. Besides, noncoding RNA is regarded as an important part of epigenetic, which can regulate gene expression at both transcription and translation level [7]. Numerous studies have shown that epigenetic processes are involved in the pathogenesis of DN. In this review, we discuss recent advances in the epigenetics of DN, including DNA methylation, noncoding RNA, and histone modifications, with the focus on the role of three types of epigenetic modification in DN.

2. DNA Methylation in DN

DNA methylation, frequently described as a "silencing" epigenetic mark, usually occurs at 5′-cytosines of CpG dinucleotide and is catalyzed by specific DNA methyltransferases [8]. Generally, if DNA methylation exists at gene promoter regions, it will lead to gene repression. On the other hand, if DNA methylation exists at gene bodies, it can modulate transcription elongation and alternative splicing. One of

the mechanisms of DNA methylation is inhibiting gene expression by methyl binding proteins, which can recruit transcriptional corepressors. Besides, another mechanism is that it can interfere with the binding of transcription factors at promoters [7].

Hyperglycemia has been reported to change DNA methylation. Moreover, in the kidney proximal tubules, diabetes is induced aberrant DNA methylation [9, 10]. However, elevated glucose level is not the only reason for maladaptive epigenetic modifications in diabetes. Many other factors can modify epigenetic profiles, such as hypoxia, inflammation, and cytokines [11, 12]. The role of DNA methylation in DN has drawn more attention, partly because of the application of high-throughput sequencing technologies. By comparing type 2 diabetes patients with or without DN, several genes have clear differential methylation. The gene UNC13B is one of them, which has been suggested to mediate apoptosis in glomerular cells as a result of hyperglycaemia and could be relevant to the initiation and pathogenesis of DN [13]. Another team identified 187 gene targets that show differentially site-specific DNA methylation in DNA extracted from the saliva of patients with type 2 diabetes and end-stage kidney disease (ESRD) when compared to diabetic patients without ESRD [14]. In microdissected tubules obtained from patients with DN, DNA methylation profiles demonstrated differentially methylated genes implicated in fibrogenesis [15]. Another study analyzed DNA methylation in kidney tubular epithelial cells and showed significance differences of methylation in 1061 genes in DN patients compared to controls [16].

Involvement of DNA methylation in DN is also supported by experimental studies. It is reported that hypermethylation of RASAL1 increased Ras activation in fibroblasts, leading to proliferation and fibrosis [17]. Exposure of vascular endothelial cells to hyperglycemia led to changes in DNA methylation at several genes involved in endothelial cell dysfunction [18]. Taken together, these researches have identified the gene targets for DN and also prove the importance of DNA methylation in regulating fibrotic and other genes associated with DN.

3. Noncoding RNAs in DN

RNA used to be considered as an intermediate molecule in protein synthesis, which is no more than a template for protein synthesis. However, with the development of high-throughput platforms, the classical view of the molecular biology has changed [19, 20]. It has been reported that less than 2% of human genome is transcribed into RNA transcripts that can code protein [21–23]. It means that most of RNAs are noncoding RNAs (ncRNAs) separated into long ncRNAs (more than 200 nucleotides in length) and small ncRNAs (less than 200 nucleotides).

4. miRNA

MicroRNAs (miRNAs) are small noncoding RNA molecules capable of silencing mRNA targets. miRNAs contain 20~22 nucleotides and typically bind to the $3'$ untranslated regions of target mRNA to promote translational repression and/or mRNA degradation [24]. It is reported that more than 2500 mature miRNAs are identified in humans and regulated at least 60% of protein-coding genes [25]. Hence, miRNAs can modulate the expression of numerous genes to alter key cellular functions and influence the course of various diseases. Some miRNAs are considered to have renal functions because they are enriched in kidney only [26]. Besides, miRNAs may have cell type and tissue-specific functions since different miRNA expression patterns were found in renal cortex and medulla [27].

Many miRNAs involved in DN have been identified [28]. Compared with nondiabetic control mice, several miRNAs (miR192, miR-200b/c, miR21, and miR-1207-5p) are upregulated in TGF-β1-treated murine mesangial cells and in renal glomeruli of mouse models of diabetes. The TGF-β1 pathway is a master regulator of renal fibrosis, which plays an important role in DN. Among these miRNAs, the best-studied one is miR-192, which has a conflicting expression in DN. miR-192 was reported to be upregulated in the glomeruli of streptozotocin- (STZ-) induced and db/db diabetic mice [29]. The upregulation of miR-192 expression is also shown in tubular cells treated with TGF-β1 [30]. However, other groups found a reduction in miR-192 in diabetic $Apoe^{-/-}$ mice, which was associated with increased fibrosis [31, 32]. Investigation results explained that these complexities are due to cell type-specific effects of miRNA and differences in the animal models studied [33], while considering that decreasing of miR-192 is not pathogenic, because Kato et al. demonstrated that miR-192 knockout (KO) mice had no kidney problem and milder kidney injury under diabetes. Moreover, some studies had reported that the decreasing of miR-192 at later stage can be just a result of nonspecific degradation of RNAs in progression of DN but not pathogenic [34, 35]. It has reported that the level of miR-192 is increased in the early stage of DN, which can be prevented via suppressing this microRNA expression in the mice model of DN [36, 37]. Many articles describe the function of miR-192 in DN. First, miR-192 can lead to upregulation of Col1a2 and Col4a1 in mesangial cells. They are key genes associated with the pathogenesis of DN [29]. Second, miR-192 can also regulate other miRNAs, such as miR-216a/miR-217 and miR-200b/c. It is known that miR-200 family members can regulate Zeb1 and Zeb2. MiR-200 can regulate collagen expression and promote the autoregulation of TGF-β1 in murine mesangial cells by inhibiting Zeb1. Moreover, miR-216/miR-217 also related to TGF-β1-induced Akt activation and cellular hypertrophy, which are considered to be a pivotal feature of DN [38]. Third, miR-192 is reported to have an amplification loop with p53 in response to TGF-β1. In this study, the levels of TGF-β1, p53, and miR192 were all increased in the renal cortex of diabetic mice compared with control ones [34]. Generally, miR-192 is one of the major regulators in the pathologic mechanism of DN.

Similarly, miR-21 is known to modulate the expression and activities of important factors related to diabetic kidney disease. The mechanism of miR-21 regulating renal injury is targeting Smad7 because downregulation of miR-21 can restore Smad7 levels and suppressed activation of the TGF-β and NF-κB signaling pathway [39]. In the renal cortex of

OVE26 type 1 diabetic mice, miR-21 was upregulated and could target Pten and promote mTOR activation, which is related to DN.

5. lncRNA

Long noncoding RNAs are defined as a large and diverse group of non-protein-coding transcripts longer than 200 nucleotides [40]. Based on the association with nearby protein-coding genes, the lncRNAs can be separated into six groups: sense (overlapping a protein-coding gene), antisense (located in antisense orientation to a protein-coding gene), bidirectional promoter (transcribed within 1 kb of promoters antisense to the protein-coding transcript), intronic (transcribed from an intron of a protein-coding gene), intergenic (between two protein-coding transcripts), and enhancer (transcribed from an enhancer region of a protein-coding gene) [41]. Circular RNAs also have been identified as they form covalently enclosed circular structure, which usually come from splicing of a protein-coding gene [42]. According to the mechanism of lncRNAs, they can be classified into four categories—signal, decoy, guide, and scaffold [43]. Accumulating evidence has demonstrated that the noncoding RNA (ncRNA) affects transcription, pre-mRNA processing, and translation [43, 44].

Unlike microRNA, there are only a few reports that have shown the relationship of lncRNAs with DN. First, as a host gene, ncRNA RP23 together with miR-216 and miR-217 were induced by TGF-β1 [45]. Another host gene ncRNA CJ241444 can be coregulated with miR-192 and is induced by TGF-β1. This mechanism involved the Smad transcription factors, protein C-ets-1, and histone acetylation [46]. Gene set enrichment analyses (GSEA) show that a megacluster of nearly 40 microRNAs and their host long noncoding RNA transcript (lnc-MGC) are coordinately increased in the glomeruli of mouse models of DN. Inhibition of the host lncRNA decreased the expression of the cluster miRNAs and attenuated early features of DN in vitro in mesangial cells and in vivo in mice [47]. Second, another study reported that 21 common lncRNAs were upregulated in wild-type, but downregulated in Smad3 knockout, kidneys in both disease models in which progressive renal inflammation and fibrosis were abolished when the Smad3 gene was deleted or suppressed [48]. An unbiased RNA-sequencing (RNA-seq) analysis of kidney glomeruli identified that lncRNA Tug1 are considered as a differentially expressed lncRNA in the diabetic milieu. This Tug1 gene is shown to regulate mitochondrial bioenergetics in diabetic nephropathy [49]. Third, one lncRNA named Malat1 may have relationship with DN. It was first identified as a prognostic marker of survival in early-stage non-small-cell lung cancers. However, the expression of Malat1 is not restricted to cancer cell, and it is ubiquitously expressed in many normal cells and tissues [50]. Diabetes is usually recognized as the vascular disease characterized by vasoregulation change, increased generation of reactive oxygen intermediates, inflammatory activation, and altered barrier function [51]. It has been reported that the MALAT1 level is significantly upregulated in diabetic animal models. Moreover, MALAT1 expression is upregulated in the kidneys of diabetic mice. The mechanism of the result is lncRNA MALAT1 inhibition attenuates the inflammatory response in condition of hyperglycaemia via serum amyloid antigen3 (SAA3) [52]. Generally, the relationship of lncRNA and DN seems an exciting emerging field that is expected to have more findings.

6. Histone Modifications in DN

Histone posttranslational modifications (PTMs) regulate gene expression chromatin structure. Important PTMs of histone include lysine acetylation (Kac), lysine methylation (Kme), and phosphorylation. They are mostly in the exposed amino-terminal tails [53]. Histone Kac is catalyzed by histone acetyltransferases such as p300 and CBP, which also act as transcription coactivators. Lysine is deacetylated by HDACs. Kme is carried out by lysine methyl transferases and demethylation by lysine demethylases [54].

Recently, some papers have shown the role of histone modifications in diabetes and diabetic complications, including DN. Histone PTMs are among the best-characterized epigenetic modifications with respect to diabetes and are clearly implicated in the reduction in the expression of genes implicated in DN [55]. The role of histone PTMs in DN has been investigated in vitro and in vivo. The functional role of histone H3 lysine methylation (H3Kme) in TGF-β1-mediated extracellular matrix (ECM) gene expression in mesangial cells has been shown under normal and high-glucose (HG) conditions [56]. Another study reported that high glucose inhibited DNA methylation and increased H3K9ac at the promoter of the redox regulating protein p66 and upregulated p66shc expression in diabetic murine kidney [57]. Histone modifications also showed connection with microRNA. Histone acetylation by p300 activated by Akt is involved in induction of miR-192 in diabetic nephropathy. These findings provide insight into the regulation of miRNAs through signaling-mediated changes in epigenetic histone acetylation under normal and disease states [46].

7. Epigenetic and ncRNA-Based Therapy

As the leading cause of renal failure, DN requires renal replacement therapy worldwide. However, effective methods to identify and halt progression of pathophysiological changes of DN remain elusive [58, 59]. In the past, proteinuria, renal pathology, and renal function are major diagnosis for DN. Recently, with the development of new technologies, such as quantitative real-time PCR, microarray, and high-throughput sequencing, it is possible for us to use ncRNA as the biomarker for DN. NcRNAs have their own characteristics. Firstly, miRNAs have consistency in particular disease and have tissue specificity. Secondly, miRNAs are highly expressed in urine and have stability storage in tissue [60, 61]. Thirdly, in some cases, miRNA changes expression level in the early stage of DN, which related to fibrosis [62]. For example, Mohan et al. reported that miR-451-5p and miR-16 in diabetes appeared to be protective against diabetes-induced kidney fibrosis, while UE miR-451-5p may hold prognostic value as an early and sensitive noninvasive indicator of renal disease [63]. Similar to miRNA, lncRNA also can help in serial

monitoring of diabetic patients. On the other hand, there are some disadvantages of using ncRNA as biomarkers to diagnose DN. With developing of renal failure, low miRNA level may be due to decreasing of synthesis capacity. So it cannot be attributed to downregulated expression.

The current standard therapy of diabetic nephropathy involves intensive treatment of hyperglycemia and strict blood pressure control, mainly via blockade of the renin-angiotensin system (RAS) [64]. As discussed earlier in this review, epigenetic regulation of DNA and histones plays important parts in DN. It has been reported that histone methylation has connection with metabolic memory. Preliminary work in endothelial cells has shown that transient episodes of hyperglycemia can induce changes in gene expression that are dependent on histone modifications and that these changes persist after return to normoglycemia [65]. Attention has been drawn to additional beneficial effects of epigenetic modifications. In connection with abnormal expression of miRNA in DN, targeting regulation of miRNA expression is regarded as a novel therapy for DN treatment.

8. Conclusions

The pathogenesis of DN involves complicated interactions between metabolic and hemodynamic factors. Increasing evidence suggests a critical role for epigenetic modifications in DN. In this review, we have highlighted some emerging mechanisms including DNA methylation, noncoding RNA, and histone modification. Rapid developments of high-throughput genome-wide screening techniques have greatly broadened the understanding of genetic and epigenetic changes in DN. Unlike genetic changes, epigenetic changes are reversible, which means it gives an opportunity for therapeutic development. However, the mechanism of action of these inhibitors is not fully clear, and more work is needed before understanding it. Epigenetic therapy has been expected to obtain more achievements in the future. Further work in this field of research has a chance to make a distinguished influence on the therapy of DN.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Mechanistic Insight and Management of Diabetic Nephropathy: Recent Progress and Future Perspective

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Diabetic nephropathy (DN) is the most serious microvascular complication of diabetes and the largest single cause of end-stage renal disease (ESRD) in many developed countries. DN is also associated with an increased cardiovascular mortality. It occurs as a result of interaction between both genetic and environmental factors. Hyperglycemia, hypertension, and genetic predisposition are the major risk factors. However, the exact mechanisms of DN are unclear. Despite the benefits derived from strict control of glucose and blood pressure, as well as inhibition of renin-angiotensin-aldosterone system, many patients continue to enter into ESRD. Thus, there is urgent need for improving mechanistic understanding of DN and then developing new and effective therapeutic approaches to delay the progression of DN. This review focuses on recent progress and future perspective about mechanistic insight and management of DN. Some preclinical relevant studies are highlighted and new perspectives of traditional Chinese medicine (TCM) for delaying DN progression are discussed in detail. These findings strengthen the therapeutic rationale for TCM in the treatment of DN and also provide new insights into the development of novel drugs for the prevention of DN. However, feasibility and safety of these therapeutic approaches and the clinical applicability of TCM in human DN need to be further investigated.

1. Introduction

Diabetic nephropathy (DN) is a major complication of diabetes and the largest single cause of end-stage renal disease (ESRD) in many developed countries. DN is also associated with an increased cardiovascular mortality. Immunologic derangement is the cornerstone of pathogenesis of CKD [1]. Of course, both primary kidney disease and secondary kidney disease dwell in this process, including DN [2]. DN is the most serious microvascular complication of diabetes and the largest single cause of ESRD in many developed countries. DN is a usual cause of ESRD in regions in which the availability of renal replacement therapy (RRT) is limited [3]. The global prevalence of diabetes is growing rashly and rapidly, especially in developing countries. Furthermore, regardless of the underlying causes of DN, the multitudinous treatments of DN do not apparently reduce its substantial morbidity and mortality and overspent disproportionate heath care expenditure and are conducted as a tremendous socioeconomic burden on society [4]. Therefore, there is urgent need for improving mechanistic understanding of DN and then developing new and effective therapeutic approaches to delay the progression of DN. This review focuses on recent progress and future perspective about mechanistic insight and management of DN.

2. The Epidemiology of DN

2.1. DN in General. DN is one of the most frequent and severe complications of diabetes mellitus and a worldwide public health problem affecting millions of people. Currently, based on the criteria of the kidney disease, Improving Global Outcomes (KDIGO) Clinical Practice Guideline, DN was diagnosed by renal biopsy or medical history [5]. DN is the largest single cause of ESRD in many developed countries and will continue to be the leading cause of death in diabetes mellitus [6, 7]. According to the data of American Diabetes Association, diabetes mellitus has a major impact on the development of DN. DN occurs in 20% to 40% of all patients with type 2 diabetes mellitus, accounting for approximately 50% of cases in the developed countries [8, 9]. In short, counting on the undiagnosed patients who do not

Table 1: Studies about reported new future therapies of DN.

Study/year	Design/numbers	Race	Endpoints
Irannejad et al., 2016 [10]	Retrospective single-center analysis, serum nesfatin-1 in patients, included 44 adult patients with type 2 diabetes and microalbuminuria and 44 control patients with type 2 diabetes and normoalbuminuria	Asians	Peripheral nesfatin-1 levels are markedly elevated in patients with type 2 diabetes and microalbuminuria
Katayama et al., 2016 [11]	Prospective multicenter-randomized analysis, the efficacy and safety of seven once-daily oral doses of finerenone, included individuals: 96	Asians	Finerenone reduced albuminuria without adverse effects on serum potassium levels or renal function
Fouad et al., 2016 [12]	Retrospective single-center analysis, the relationship between serum uric acid and hypertension in DN, included individuals: 986	Caucasians	Serum uric acid level may identify and link with the onset of hypertension in DN
Machingura et al., 2017 [13]	Prospective cross-sectional analysis, prevalence of and factors associated with DN in Zimbabwe, included individuals: 344	Blacks	Prevalence of DN is higher in type 1 and type 2 diabetes mellitus patients than previously reported in Zimbabwe
Perkowska-Ptasinska et al., 2016 [14]	Retrospective multicenter analysis, biopsy based data from 14 renal centers in Poland, included individuals: 352	Caucasians	The relatively high prevalence of potentially curative kidney diseases of renal biopsy in these patients
Kaidonis et al., 2016 [15]	Prospective multicenter analysis, the single nucleotide polymorphism (SNP) rs2910164 residing within microRNA-146a (miR-146a) is associated with DN, included individuals: 890	Caucasians	Rs2910164 is significantly associated with microvascular complications DN
Li et al., 2015 [16]	Prospective multicenter randomized analysis, the additional benefit and safety of the Chinese herbal granule Tangshen Formula (TSF) in treating DN, included individuals: 180	Asians	TSF appears to be a safe therapeutic treatment for DN patients

yet realize their condition, the overall prevalence of DN is unoptimistic. Particularly, Table 1 summarizes selected studies that evaluated DN incidences and outcomes in diabetic nephropathy patients and also are related to the topic [10–16]. American Diabetes Association accurately defined diabetes mellitus as "a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both" [17]. And it is dreadful because of the complications including long-term dysfunction and failure of different organs.

2.2. DN in China. The prevalence of DN has been dramatically increased in recent decades; especially in China, the adjusted prevalence of diabetes mellitus and prediabetes among Chinese adults is 9.7% and 15.5%, respectively [18, 19]. In China, DN is growing at an alarming rate. A national survey which started by Peking University Institute of Nephrology indicates that China is suffering from afflictions associated with increasingly unhealthy diets and obesity. Besides a rise in the incidence of diabetes, the growth rate of CKD has been surpassed in the United States, although the number of Chinese citizens with CKD dwarfs the number in the United States [20]. The situation of China on the whole is that, owing to the lack of financial and clinical resources, as well as inequalities in access to across all regions and populations, the prevention and management of DN is unfulfilled in the last few decades. But with the advocacies and endeavors of the government and the Chinese Society of Nephrology (CSN) increases, the high efficiency health care system has covered almost all the population, and patient care and medical education in the field of DN have been greatly improved in the past years [21]. The currently available treatment improves the conditions of DN patients but still cannot completely stop the progression of DN to ESRD. Therefore, there is an urgent need for the development of novel therapeutic approaches for the treatment of DN.

3. The Pathophysiological Process of DN

DN occurs as a result of interaction between both genetic and environmental factors. Hyperglycemia, hypertension, and genetic predisposition are the major risk factors. The aggravating hyperglycemia is also a high risk factor for the development of microvascular complications [22]. In other words, patients with diabetes mellitus tend to have increased the risk of developing microvascular complications, especially organs with arterioles and microvessels, such as eyes and kidney, which causes horrible retinopathy and nephropathy [23]. The pathogenesis of the microvascular complications of diabetes mellitus is not yet fully understood, but hyperglycemia always acts as an initiating and sustaining factor to continuously damage target tissues and organs. Tissues susceptibility increases as a result of significant microvasculopathy and of interstitial inflammation. Because kidney is an organ with high bioenergetic needs, hyperglycemia with its glucotoxicity makes arterioles and microvessels hyaline degeneration and fibrinoid degeneration which seems to be triggering a vicious cycle and causes damage to renal mitochondria resulting in bioenergetic deficit of renal tubular epithelial cells [24].

Podocyte damage occurs at a relatively early stage in CKD [25]. Podocyte is a terminal differentiated glomerular epithelial cell which locates the outside of the glomerular

basement membrane (GBM) and as the final barrier of glomerular filtration barrier, podocyte leads a crucial role in establishing the selective permeability of the glomerular filtration barrier [26, 27]. Severe podocyte injury could appear in DN, especially the fusion of podocyte foot processes (FPs). Such phenomenon may explain why podocyte injury is typically associated with marked proteinuria in DN [28]. Furthermore, in the research of multiphoton excitation fluorescence microscopy of the filtration barrier, they have further visualized a new information on intact podocyte and also provided an additional multiphoton evidence for the glomerular origin of proteinuria by imaging the morphologic and functional consequences of localized podocyte damage using the experimental model of FSGS and localize the areas of podocyte damage [29]. Hence, the concept of podocyte loss or detachment as an important factor in the glomerulosclerotic process in diabetes is well documented. The promise of this new insight is the development of new and effective strategies for the prevention and treatment of DN.

4. Prevention and Management of DN

4.1. The Present Therapies. The management of diabetes mellitus hinges on "five carriages," which are health education, diet, exercise, weight control, and drug treatment, respectively, of which the former four therapies are described as nonpharmacological measures. And the pharmacological agents currently approved for treatment of diabetes mellitus include sulfonylureas, metformin, acarbose, and insulin [30]. Nonpharmacological measures are critical to the early stages of diabetes mellitus, but people who often ignore the importance of nonpharmacological measures are often ignored, while pharmacological measures are the most frequently used therapies at this stage and the goal would be to have a drug ameliorate or correct both of these abnormalities in the patient with diabetes mellitus. Fortunately, multitudinous research continues to furnish advanced understanding of the pathophysiology and outcomes of this disease, which could help patients to change views and to control diabetes mellitus better with proper diet, applicable exercise, and advisable weight control [31]. All of these are the principles of management of diabetes; management of DN could be fit for the same principles. Now, the principles of the management of DN could be summarized in three parts: glycemic control, management of proteinuria, and intervention of merging symptoms.

4.1.1. Glycemic Control. There is no doubt that glycemic control plays the most momentous role in management of DN; keeping blood glucose within normal limits is a foundation and prerequisite for the treatment of DN [32]. Several studies have indicated that a close relationship has been established between poor glycemic control and microvascular complications, including DN [33]. And many studies have confirmed that strict glycemic control could generate a beneficial effect to slow down the progression of DN and significantly decrease albuminuria in trials [34]. According to many previous studies, patients with diabetes mellitus have been shown to have defects in sensitivity of target organs and tissues to insulin and to relatively inadequate insulin output [35]. Based on these, there are two ways to correct hyperglycemia: one is increasing sensitivity of target organs and tissues to insulin and another is stimulating insulin secretion, corresponding to the clinical commonly used first-line drugs thiazolidinediones of which representative drug is troglitazone and sulfonylureas, respectively. Troglitazone is an antidiabetic antihyperglycemic agent; tons of research of troglitazone have been studied for more than a decade [36]. As one of the only commonly used drugs, troglitazone exploits tissue sensitization to insulin as the main mechanism of action and exert a furthersome effect on β-cells function of pancreatic island [37, 38]. Hence, the risks of diabetic complications could be reduced. Secondly, the sulfonylureas have been regarded as the primary drug for treatment of diabetes mellitus for decades, and the dominating function of sulfonylureas is stimulate insulin secretion by β-cells of pancreatic island [39]. Moreover, sulfonylurea therapies could lower the blood glucose levels and raise plasma insulin levels in the untreated patients with diabetes mellitus and postprandial blood glucose levels and low plasma insulin levels [40]. Sulfonylureas augment insulin secretion has no direct actions on insulin sensitivity which is totally different with thiazolidinediones [41]. In addition, there are two types of drugs commonly used in daily clinical aside from thiazolidinediones and sulfonylureas, metformin, and acarbose. They are distinguished very effectively in lowering blood glucose in patients with diabetes mellitus with minimal side-effects; besides, studies have found that metformin also could exert renoprotective properties owing to its multieffects on multiple signaling pathways apart from its use as an antidiabetic drug [42–44]. Taken together, despite the benefits derived from strict control of glucose, many patients continue to enter into ESRD. Thus, there is urgent need for the development of new and effective therapeutic approaches to prevent DN.

4.1.2. Management of Proteinuria. Proteinuria is considered as a hallmark and sensitive marker of DN, which is mainly caused by the severe podocyte injury [45]. But what could we do to deal with proteinuria in clinic now? Conventional angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) is recommended for the management of DN, especially for patients of DN with high blood pressure [46]. They blocked the renin-angiotensin-aldosterone system (RAAS) pathway, which is the most important component in the development and progression of DN [47]. In addition, a breakthrough of emerging evidence shows that the use of mineralocorticoid receptor blockers (MRB) in combination with ACEI or ARBs might have benefits on proteinuria [48]. In conclusion the long-term clinical use of ACEI or ARB has been proven safe and effective but still needs to be used with caution in those with decreased renal function, especially with severe kidney failure.

4.1.3. Intervention of Merging Symptoms. At the same time, many patients of DN still show merging symptoms, such as dyslipidemia and hypertension. Poorly controlled blood glucose levels in patients with DN are associated with dyslipidemia which constitutes an additional risk factor.

Dyslipidemia consists of elevated levels of low density lipoprotein cholesterol and low levels of high-density lipoprotein cholesterol and increased levels of apolipoproteins B [49]. In view of this, statins, 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are recommended for DN with normal low density lipoprotein levels as well and have a major role in preventing another long-term complications in diabetes mellitus [50]. Fortunately, these abnormalities could also be improved by favorable control of blood glucose levels [51]. Hypertension is a common comorbidity usually following the development of DN, several multiple prospective randomized placebo-controlled trials demonstrate that tight blood pressure control among patients with DN could decrease the rates of macrovascular complications as well as microvascular complications [52]. Given the pathogenesis and clinical symptoms of DN with hypertension, ACEI or ARB therapies could slow nephropathy progression which means that the hallmark of very high proteinuria of DN could have a great relief while restoring kidney function in the meantime [53]. Hopefully, new therapeutic strategies and directions of these present therapies could be explored through our unremitting efforts with the advance in the understanding of DN.

5. The Future Therapies of DN

Cutting-edge evidence shows that there are certain trends of the future therapies for DN, including comprehensive exploration of existing medicines, application of molecular biology discoveries, progress in stem cell therapy, and the applicability of traditional Chinese medicine (TCM) in the treatment of DN.

5.1. Comprehensive Exploration of Existing Medicines. As mentioned previously, The RAAS pathway is very important in the progression of DN. Several studies indicate that the combination of spironolactone with an ACEI or ARB could significantly mitigate proteinuria compared with the placebo [47, 54]. Furthermore, there are evidences which indicate that lack of a vitamin D receptor (VDR) which is a member of the nuclear receptor superfamily and acts as an ordinary nuclear receptor hormone could directly but not exclusively regulate gene transcription, resulting in the induction or suppression of vitamin D target genes which resulted in an increase in RAAS activity and significant proteinuria [55, 56], which means 1,25(OH)2D3 that exert renal protective effects and might involve suppression of renin gene transcription as well as high glucose-induced angiotensinogen production [57]. By the way, research reported that 1,25(OH)2D3 could reduce the hypercorrection of fibroblast growth factor 23 (FGF23) level which is also a risk factor for the progression of DN and could damage podocyte [58]. These studies strongly supported the recommendation of vitamin D supplementation for the prevention and management of DN.

5.2. Application of Molecular Biology Discoveries. Studies performed have discovered several key signaling pathways in succession, such as endoplasmic reticulum (ER) stress, which already have become a therapeutic target of much concern for DN [59]. Besides, there are researches of the glucagon-like peptide-1 (GLP-1) which are secreted by the intestinal enteroendocrine cells in response to ingestion of various nutrients showing that GLP-1 can bind to β-cell receptors, stimulate insulin release, and improve glycemic control and then play a protecting role in management of DN [60]. Molecular biology has a great development in decades, especially in the 21st century; in recent years, studies of nuclear factor kB (NF-kB) which has been postulated in many immune systems and inflammation are on fire. NF-κB is a ubiquitous nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of inflammation [61]. The concrete mechanisms are that NF-kB promotes the expression of a number of genes involved in inflammation, such as cytokines, and activates apoptosis process, and might be able to translate the therapeutic potentials for DN into reality [62].

5.3. Progress in Stem Cell Therapy. Stem cells that are identified nearly half a century ago exhibited great potential for the repair of damaged tissues and organs and provided new hope for a means to change the track of DN [63]. Multiple preclinical studies have demonstrated that the innovative stem cell therapy could also be used in kidney, such as the using of mesenchymal stem cells (MSCs) of late years [64]. MSCs are undifferentiated cells capable of self-renewal and multilineage differentiation. Interestingly, the administration of MSCs could prevent renal injury and promote renal recovery through a series of complex mechanisms and on account of the therapeutic potentials. Therefore, MSCs are being evaluated as a possible member in treatment of DN [65]. Apoptosis of stem cells is likely to promote eventual manifestation of kidney failure in diabetes mellitus [66]. Furthermore, the present study shows reductions in subsets of stem cells in peripheral blood and renal cell preparations of db/db mice which is a model of DN, which indicates that the decrease of stem cells might be a hallmark of DN [66, 67]. MSC transplantation is a promising therapeutic strategy to delay DN progression in animal models; however, the clinical trials should be performed to investigate the safety and efficacy of MSC transplantation in patients with DN [68]. Overall, stem cells could be used as an early diagnosis marker of DN and have a tendency to play much more important roles in the future.

5.4. Further Research about Traditional Chinese Medicine (TCM). TCM has long histories in China and has emerged and influenced hundreds of thousands of people. Historically, conventional medicines, such as ACEI or ARB, are not able to totally prevent the development of DN [16]. Thus, there is an urgent need to find new effective agents to delay the progression of DN. TCM could produce prominent effects and there are several expert consensuses and recommendations for the treatment of CKD by improving proteinuria, such as Tripterygium and Emodin [69, 70]. Nowadays, an increasing understanding and popularity of TCM caused great interests in lots of diseases on its efficiency and mechanisms by the spring up of molecular biology, especially the application of Ultra Performance Liquid Chromatography (UPLC) and

mass spectrometer which could analyze the active ingredients of TCM and manifest herbal medicine as western medicine. For the past few years, notoginsenoside R1 could retard DN by ameliorating podocyte adhesion through $\alpha 3\beta 1$ integrin upregulation and astragaloside IV could inhibit podocyte apoptosis by downregulation of PERK-ATF4-CHOP pathway [71, 72]. Furthermore, there is a present study that aimed to evaluate the effect of ARB combined with Chinese formula Qidan Dihuang Grain (QDDHG) in improving proteinuria of patients of DN [73]. Yiqi Yangyin Huoxue Method is also a valid complementary and alternative therapy in the management of diabetic nephropathy, especially in improving UAER, serum creatinine, fasting blood glucose, and beta-2 microglobulin [74]. These findings strengthen the therapeutic rationale for TCM in the treatment of DN and also provide new insights into the development of novel drugs for the prevention of DN. However, the clinical applicability of TCM in human DN needs to be further investigated.

6. Controversy in Current Therapy of DN

It is a long time that the relationship between hypertension and DN has been the subject of controversy, and after diabetes mellitus, hypertension is the second most commonly reported etiology of ESRD in the United States Renal Data System, and hypertension treatment targets in patients with DN remain important clinical concerns [75]. Recent study demonstrated that the association between the renin gene polymorphism and the risk for developing DN in patients with type 1 diabetes may solve this problem in the future [76]. Angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II receptor antagonists (ARB) are widely used in DN; however, blockage of the rennin-angiotensin system may not completely delay disease progression. Thus, there are high priorities to develop new and effective therapeutic approaches to prevent the progression of DN.

7. Conclusions

Despite the benefits derived from strict control of glucose and blood pressure, many patients continue to enter into ESRD. Thus, there is urgent need for improving mechanistic understanding of DN and then developing new and effective therapeutic approaches to delay the progression of DN. Many studies strengthen the therapeutic rationale for TCM in the treatment of DN. However, feasibility and safety of these therapeutic approaches and the clinical applicability of TCM in human DN need to be further investigated.

Competing Interests

No potential conflict of interests relevant to this article was reported.

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Review Article

Classification and Differential Diagnosis of Diabetic Nephropathy

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Diabetic nephropathy (DN) is a major cause of end-stage renal disease throughout the world in both developed and developing countries. This review briefly introduces the characteristic pathological changes of DN and Tervaert pathological classification, which divides DN into four classifications according to glomerular lesions, along with a separate scoring system for tubular, interstitial, and vascular lesions. Given the heterogeneity of the renal lesions and the complex mechanism underlying diabetic nephropathy, Tervaert classification has both significance and controversies in the guidance of diagnosis and prognosis. Applications and evaluations using Tervaert classification and indications for renal biopsy are summarized in this review according to recent studies. Meanwhile, differential diagnosis with another nodular glomerulopathy and the situation that a typical DN superimposed with a nondiabetic renal disease (NDRD) are discussed and concluded in this review.

1. Introduction

Diabetic nephropathy (DN) caused by diabetes mellitus is one of the major causes of end-stage renal failure worldwide [1]. Clinically, microalbuminuria is an important index to assess the progression of DN [2]. However, it is not accurate to evaluate the severity or prognosis simply based on the degree of proteinuria. It is now well recognized that not all diabetic patients who develop renal function failure have massive albuminuria [3]. Therefore, nephrologists and endocrinologists should be aware of the significance of pathological changes of DN in their clinical practice. Specifically, nondiabetic renal disease (NDRD), which might commonly be superimposed with diabetic renal lesions in some patients with type 2 diabetes, could only be confirmed and excluded by biopsy [4].

2. Pathological Changes of DN

The most significant and consistent pathological changes identified in renal biopsies of clinical DN patients are the glomerular lesions [5] which are, especially, diffuse and nodular mesangial expansion and Glomerular Basement Membrane (GBM) thickening [6]. Diffuse mesangial expansion, which develops at as early as 5th year since the onset of diabetes, is the earliest observable change by light microscopy [7]. The mesangial fractional volume [Vv(Mes/glom)] is correlated with albumin excretion rate (AER) and Glomerular Filtration Rate (GFR) in both type 1 [8] and type 2 diabetes [9]. As the disease advances, diffuse mesangial expansion progressively develops into nodular accumulations of mesangial matrix in the late stage of the DN. These nodular lesions, also known as Kimmelstiel-Wilson nodules, can be observed in about 25% of patients with advanced DN [10]. Nodular lesions and diffuse lesions are two stages of DN. Compared to the patients with diffuse mesangial expansion, those patients with nodular diabetic glomerulosclerosis present more severe renal damage, longer diabetic durations, and poorer renal prognosis [11].

GBM thickening can be observed within 2–8 years after the onset of diabetes. It is an early lesion which could be detected and measured by electron microscopy (EM) [12]. GBM width tends to increase linearly according to diabetes

Table 1: Diabetic nephropathy is divided into four hierarchical glomerular lesions.

Class	Description and criteria
I	Mild or nonspecific changes on light microscopy and conformed GBM thickening proven by electron microscopy: GBM > 395 nm (female), GBM > 430 nm (male).
IIa	Mild mesangial expansion in >25% of the observed mesangium; area of mesangial proliferation < area of capillary cavity.
IIb	Severe mesangial expansion in >25% of the observed mesangium. Area of mesangial proliferation < area of capillary cavity.
III	At least one convincing nodular sclerosis (Kimmelstiel-Wilson lesion).
IV	Advanced diabetic glomerulosclerosis in >50% of glomeruli.

Table 2: Separate scoring system of interstitial and vascular lesions of DN.

Lesion	Criteria	Score
Tubulointerstitial lesions	No IFTA	0
	IFTA < 25%	1
	25% < IFTA < 50%	2
	IFTA > 50%	3
Interstitial inflammation	Absent	0
	Relate to IFTA	1
	In areas without IFTA	2
Arteriolar hyalinosis	Absent	0
	One hyaline arteriole	1
	More than one hyaline arteriole	2
Arteriosclerosis (most severely affected artery)	No intimal thickening is observed	0
	Intimal thickening is less than the thickness of the media	1
	Intimal thickening is more than the thickness of the media	2

Note. IFTA: tubulointerstitial fibrosis and tubular atrophy.

duration in type 1 diabetes [13]. Vv(Mes/glom) and GBM width together explain 59% of the AER variability in a group of 125 patients of type 1 diabetes [8].

Although diabetic glomerular lesions have been the focus of the investigation on DN, the extraglomerular lesions are also involved in the progression of the disease. Tubulointerstitial lesions, including tubular atrophy, interstitial inflammation, and tubulointerstitial fibrosis, are closely related to renal function loss in the progression towards ESRD in patients with preexisting renal insufficiency [14]. Since DN is a kind of diabetic microangiopathy, hyalinosis occurs in both afferent and efferent arterioles. The hyalinosis of the efferent arteriole is a typical lesion by which diabetic nephropathy could be differentiated from hypertensive nephropathy [15].

There is increasing recognition of lesions like glomerular endothelial injury [5], podocyte impairment [16], and glomerulotubular junctions abnormalities in DN [17]. Given that the detection methods of these lesions are difficult to generalize in clinical practice, for now their value in diagnosis and classification is not as important as glomerular, tubulointerstitial, and vascular lesions.

3. Tervaert Classification of Diabetic Nephropathy

Journal of the American Society of Nephrology published pathological classification of DN by Tervaert et al. in 2010 (Tables 1 and 2) [10].

4. The Application and Evaluation of Tervaert Classification of DN

4.1. Contribution to the Early Diagnosis of Diabetic Nephropathy and Guiding Significance for Renal Prognosis in Patients with Diabetes. The classification outlined in Tervaert et al. is based on glomerular lesions, which best reflect the course of progressive DN. This is an important first step to set up an evaluable scheme of clinical value. Patients with typical DN show a longer impairment duration, worse metabolic control, and higher prevalence of diabetic retinopathy [18]. Diabetic patients with microalbuminuria without glomerulopathy are more likely to either regress to normoalbuminuria or remain

with microalbuminuria with a slower rate of decline in renal function [19].

Using Tervaert pathological classification, Zhu et al. rediagnosed 37 cases of renal biopsies obtained from patients with type 2 diabetes manifesting microalbuminuria or clinical albuminuria and found that 6 out of 11 patients who were previously diagnosed with nondiabetic nephropathy actually belonged to class I~class II DN [20]. To explore the significance of this classification on prognosis, one group analyzed the relationship between structural changes and clinical features in 50 patients with type 2 diabetic and found that, as the glomerular lesions advanced from classes I to IV, the average GFR and 5-year renal survival decreased (100% of renal survived in classes I and IIa patients and 75% and 66.7% in classes IIb and III patients, respectively, whereas only 38.1% in class IV patients) [21]. Furthermore, another large-scale follow-up study of 396 patients with type 2 diabetes also revealed that the severity of glomerular and interstitial lesions had a significant impact on renal prognosis and could be used as an independent risk factor for renal outcomes [22]. Studies mentioned above all employed Tervaert pathological classification of DN, which suggested that this method can help to increase the accuracy of diagnosis, the possibility of an early diagnosis, and treatment of DN, as well as the prediction of renal prognosis.

As interstitial lesions also contribute to the impairment in renal function and may be an independent factor involved in the progression of DN, the classification scheme has put the extraglomerular lesions into consideration and introduces a separate scoring system for interstitial and vascular lesions.

4.2. Requirements to Involve Tubular, Interstitial, and Vascular Lesions into the Classification. Although the severity of tubular, interstitial, and vascular lesions has been taken into consideration by Tervaert classification, it is still not enough to meet practical requirements. Before Tervaert classification, Fioretto classification has been used for a long time, which included tubular, interstitial, and vascular lesions and divided DN into 3 categories according to the pathological changes under light microscope: C1, normal/near normal; C2, typical diabetic nephropathy with predominantly glomerular changes; and C3, atypical patterns of injury, associated with disproportionately damage including tubulointerstitial or arteriolar hyalinosis and with absent or only mild diabetic glomerular changes [23]. By using Fioretto classification, 31 patients with type 2 diabetes accompanied with normo-, micro-, or macroalbuminuria were investigated, and GFR lower than 60 mL/min/1.73 m^2 was considered as the decline of renal function [24]. Majority of patients (22 of 23) with micro- or macroalbuminuria were diagnosed as typical glomerular changes (C2) of Fioretto classification, and only one patient was diagnosed as atypical pattern of renal damage (C3). Notably, among patients with normoalbuminuria, 3 of 8 were diagnosed as C3 according to the tubular, interstitial, and vascular lesions [24]. When compared to Fioretto classification, Tervaert classification was only based on glomerular lesions, which is not sufficient for clinical application, which failed to classify tubular, interstitial, and vascular lesions belonging to Fioretto C3, due to the heterogeneity of the renal lesions and the complicated mechanism underlying diabetic nephropathy.

4.3. The Complexity and Heterogeneity of Type 2 Diabetes Should be Involved in Classification. Renal lesions in type 2 diabetes are much more complex than those in type 1 diabetes. There are more challenges to reach a correlation or predictive accuracy in renal function with glomerular structural variables in type 2 diabetes than in type 1 diabetes [19]. Tervaert et al. suggested that their classification can be used for both type 1 and type 2 diabetic patients, because of the substantial overlaps between these two types in histologic changes and clinical complications [10]. However, failure to distinguish the patients with type 1 diabetes or with type 2 diabetes in Tervaert classification might limit its significance on clinical practice [25]. There are two points worth taking into account for type 2 diabetes.

The first point is the high prevalence of nondiabetic superimposed renal lesions in type 2 diabetes. In many clinical cases, renal biopsies are usually performed in patients with an atypical manifestation of DN. There is no wonder that nondiabetic renal lesions in proteinuric type 2 diabetic patients have a prevalence as high as approximately 30% [18].

The second point is the heterogeneity in renal structure and pathogenesis of type 2 diabetes. Due to the heterogeneity of type 2 diabetes, a minority of type 2 diabetic patients have typical histopathological patterns resembling those present in type 1 diabetes, due to the heterogeneity of type 2 diabetes. Only 30% of type 2 diabetic patients with microalbuminuria and 50% of patients with proteinuria demonstrate typical diabetic glomerulopathy [23]. Atypical patterns of renal injury account for 35% of those with microalbuminuria and proteinuria. These patients exhibiting mild or atypical diabetic glomerular lesions usually present severe tubulointerstitial lesions which include tubular atrophy, TBM thickening and reduplication, advanced glomerular arteriolar hyalinosis associated with atherosclerosis of large vessels, interstitial fibrosis, and global glomerular sclerosis. These tubulointerstitial lesions not only are related to hyperglycemia, but also reflect the contributions from various causes predated type 2 diabetes, such as ageing, atherosclerosis, and systemic hypertension [23].

5. Differential Diagnosis of DN with another Nodular Glomerulopathy

Nodular diabetic glomerulosclerosis has a variety of pathological features but still should be differentiated from another mesangial nodular sclerosing glomerulopathy, which usually has similar light microscopic manifestations. It is necessary and essential to include manifestations, immunofluorescence staining (IF), and EM into the distinction of lesions caused by immune complex or monoclonal protein [26].

Nodular lesions could be observed in various renal primary and secondary diseases, such as membranoproliferative glomerulonephritis, renal amyloidosis, type III collagen glomerulopathy, monoclonal immunoglobulin or light chain deposition disease, fibronectin nephropathy, and cryoglobulinemia glomerulosclerosis [6, 27–29]. Table 3 shows the

Table 3: Differential diagnosis of DN with another nodular glomerulopathy.

Disease	Clinical manifestations	Light microscopy	Immunofluorescence microscopy	Electron microscopy
Diabetic nephropathy	Long duration of diabetes	Mesangial nodular sclerosis; PAS (+); silver (+)	Linear deposition of immunoglobulin (Ig) G, with or without IgM and C3 in sclerotic nodules	Mesangial expansion; diffuse GBM thickening; nonspecific fibrillar deposition
Membranoproliferative glomerulonephritis	Chronic nephritis, nephrotic syndrome, hypertension, hypocomplementemia	Mesangial nodular sclerosis; mesangial insertion; double contouring; PAS (+); silver (+)	Granular deposition of multiple immunoglobulin deposition and complement components	Subendothelial (type I), intramembranous, often ribbon-like or nodular (type II), subepithelial (type III) electron-dense deposits
Renal amyloidosis	Chronic infection, systemic amyloidosis, lymphoproliferative disease, long-term dialysis, family inheritance	Mesangial nodular sclerosis; Congo red (+)	Light chain λ (+) in the mesangium, GBM, tubulointerstitium, and blood vessel wall	Amyloid fibrils (randomly oriented, nonbranching, 9–11 nm in diameter)
Monoclonal immunoglobulin/light chain deposition disease	Ageing, plasma cell dyscrasia, idiopathic	Mesangial nodular sclerosis; PAS (+); silver (−)	Monoclonal light chain κ (+) in the GBM, TBM, and vascular wall basement membranes	Granular, powdery deposits
Type III collagen glomerulopathy	Persistent proteinuria, nephrotic syndrome	Mesangial nodular sclerosis; PAS (weak +)	Collagen III (+)	Parallel collagen fibers (100 nm in diameter)
Fibronectin nephropathy	A rare autosomal dominant disease, nephrotic syndrome	Mesangial nodular sclerosis; PAS (+); Congo red (−)	Fibronectin (+)	Granular deposits with short fibers (10–14 nm in diameter)
Cryoglobulinemia glomerulosclerosis	Proteinuria, nephrotic syndrome, high serum cryoglobulins, lymphoproliferative disorders	Intracapillary proliferation and inflammatory cell infiltrates, intracapillary thrombi, nodular glomerulosclerosis; double contouring; PAS (+)	Monoclonal or polyclonal immunoglobulin (IgM, IgG and C3), rheumatoid factor	Organized electron-dense deposits (microtubular, 30 nm in diameter)
Idiopathic nodular glomerulosclerosis	Smoking, long-standing hypertension; normal glucose metabolism	Similar to those of nodular diabetic glomerulosclerosis,	IgG and albumin	No electron-dense or fibrillar deposits

differential diagnosis of several mesangial nodular sclerosing glomerulopathy based on clinical manifestations, periodic acid Schiff (PAS) stain, methenamine silver stain, IF, and EM.

6. DM Accompanied with Nondiabetic Renal Disease (NDRD)

DM patients generally do not receive renal biopsy, unless there is a need to make an exclusive or differential diagnosis due to the complicated clinical situation. Because most of the DM patients undergoing renal biopsy show compound clinical manifestations, it is easy for pathologists to find superimposed lesions in renal biopsy in addition to pure pathological changes of DN.

Normally, no immune complexes and obvious complements could be detected by IF and EM in patients with DN. If there is a variety of typical deposition in the glomeruli, such as granular/chunky patterns of immunoglobulin by IF or electron dense deposits by EM, it usually indicates a superimposed nondiabetic renal disease [6].

In China, IgA nephropathy was the most frequently biopsy finding seen in all NDRD patients, followed by membranous nephropathy, mesangial proliferative glomerulonephritis, hypertensive nephrosclerosis, renal damage, minimal-change disease, focal segmental glomerulosclerosis, and crescentic glomerulonephritis [30]. However, the disease spectrum of NDRD varies in different populations. For example, in the United States, unlike in China which has a high prevalence of IgA nephropathy, two large-scale retrospective studies found that focal segmental glomerulosclerosis, acute tubular necrosis, and IgA nephropathy were the most common lesions found in patients with NDRD. Hypertensive nephrosclerosis, minimal-change disease, and membranous nephropathies were also common NDRDs of diabetic patients in the United States [31, 32]. In this review, we focus on the prevalence of NDRD in China. Table 4

Table 4: Literature review of NDRD in China.

Author	Year of publication	References	Number of patients	Pathological diagnosis			Statistical analysis of NDRD (alone and coexistent with DN)
				DN	NDRD	DN plus NDRD	
Mak et al.	1997	[35]	51	67	16	17	IgAN (59%), HTN (24%)
Wong et al.	2002	[36]	68	35	46	19	IgAN (29.5%), HTN (20.4%), MN (18.2%), MCD (9%)
Cao et al.	2007	[37]	120	85.8	—	14.2	IgAN (41.2%), MN (17.6%), HTN (11.7%), TIN (11.7%), RA (5.9%), FSGS (5.9%), micropolyarteritis (5.9%)
Zhou et al.	2008	[38]	110	54.5	—	45.5	IgAN (34%), MN (22%), MPGN (14%), HBV-associated GN (8%), MCD (4%), HTN (4%), MPGN (4%), CrGN (2%)
Lin et al.	2009	[39]	50	48	22	30	MN (19.23%), IgAN (11.54%), MCD (3.85%), HTN (3.85%), ATN (3.85%), CIN (7.69%)
Mou et al.	2010	[40]	69	47.8	52.2	—	FSGS (37.7%), IgAN (15.9%), MCD (15.9%), MN (8.7%)
Bi et al.	2011	[41]	220	54.5	—	45.5	IgAN (34%), MN (22%), MPGN (14%), HBV-associated GN (8%), MCD (4%), HTN (4%), MPGN (4%), CrGN (2%)
Zhang et al.	2011	[42]	130	73.9	26.1	—	IgAN (16.9%), MN (6.15%)
Zhuo et al.	2013	[43]	216	6.5	82.9	10.7	17–35 years (group I): IgAN (29%), MN (11.8%), FSGS (8.8%), MPGN (8.8%), APGN (5.9%), CrGN (5.9%); 36–59 years (group II): IgAN (34.7%), MN (15%), FSGS (1.4%), MPGN (6.1%), APGN (0.7%), CrGN (1.4%); 60 years (group III): IgAN (2.9%), FSGS (2.9%), MN (25.7%), CrGN (8.6%), MPGN (11.4%), RA (5.7%)
Peng and Wang	2013	[44]	61	52.5	—	47.5	IgAN (31%), MN (17.2%), MPGN (13.8%), HTN (13.8%), FSGS (10.3%), MCD (6.9%), APGN (3.5%)
Wang	2015	[45]	56	57.1		42.9	IgAN (33.3%), MN (25.0%), MPGN (20.8%), HTN (8.3%), FSGS (4.2%), MCD (4.2%)

Note. IgAN, primary IgA nephropathy; HTN, hypertensive nephrosclerosis; MN, membranous nephropathy; MCD, minimal change disease; TIN, tubulointerstitial nephritis; RA, renal amyloidosis; MPGN, mesangial proliferative glomerulonephritis; CrGN, crescentic glomerulonephritis; ATN, acute tubular necrosis; CIN, chronic interstitial nephritis; FSGS, focal segmental glomerular sclerosis.

summarizes recent studies on NDRD which include both NDRD alone and alongside concomitant DN in China.

As the DN and NDRD have different causes, their relationship, synergistic or independent, remains to be further studied. Given the fact that there is a wide clinical variation of DN patients combined with NDRD, the renal biopsy is an important method to improve the detection rate of NDRD. A clear diagnosis of the renal disease and proactive treatment meanwhile can stabilize or even reverse the renal function and improve the long-term prognosis of patients.

7. The Indications for Renal Biopsy

Although renal biopsy is the gold standard of DN diagnosis, the majority of diabetic patients with renal involvement are not biopsied. Some scholars believe that most diabetic glomerular changes are nonspecific in the early stage of diabetes, so there is no need to expand the indications of renal biopsy blindly. The bleeding risk of renal biopsy should be carefully considered in patients who have been suffering from hypertension, renal dysfunction, or anemia [33].

Moreover, microalbuminuria is clinically considered as a major index to judge the progression of DN. However, it is not as accurate as expected. For example, type 1 diabetes usually develops into DN within 10 to 15 years after diagnosis, while microalbuminuria may occur as early as 2 to 5 years after diagnosis. Some patients with type 2 diabetes may already have microalbuminuria at the time of diagnosis, but without DN. Thus, renal biopsy and morphological changes may offer important insights into the understanding of the complex course of diabetes and help to classify, diagnose, prognose, and manage the disease. Indications for renal biopsy in DN are as follows [30, 34]:

(1) Proteinuria of nephrotic range with diabetes less than 5 years or normal kidney functions

(2) An unexplained microscopic hematuria (especially acanthocytosis and cellular cast)

(3) An unexplained rapidly worsening renal function in patients with a previously stable renal function

(4) Application of angiotensin converting enzyme inhibitor (ACEI)/angiotensin receptor antagonist (ARB) for 2~3 months, while GFR decreased by more than 30%

(5) Failure to exclude renal diseases in the absence of diabetic retinopathy, with or without systemic diseases

8. Conclusion

DN has a pathological diversity and affects all structural components of the kidney. Recognition of these lesions and their morphological characteristics in renal biopsy may aid in preventing, slowing down, or even reversing the processes of diabetic nephropathy. Tervaert classification of DN has a positive meaning in developing novel strategies for an early diagnosis and treatment of DN. However, the complexity and heterogeneity of type 2 diabetes, different from type 1 diabetes, along with tubular, interstitial, and vascular lesions should be taken into consideration in new classification methods in the future. Regardless of its limitations, the Tervaert classification represents a very meaningful step towards the establishment of a DN classification scheme with clinical utility. When the pathologist observes the pathological changes of DN, they still need to make a differential diagnosis with another nodular glomerulopathy and clarify whether it is a typical DN complicated with NDRD or not. Furthermore, the indications and risks of renal biopsy should be prudently taken into consideration.

Competing Interests

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

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Research Article

Investigating Factors Associated with Depressive Symptoms of Chronic Kidney Diseases in China with Type 2 Diabetes

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Aim. To assess the depressive symptoms status of chronic kidney diseases in Nantong, China, with type 2 diabetes and to identify factors associated with depressive symptoms. *Methods.* In this cross-sectional analytic study, 210 type 2 diabetic patients were recruited from the Second Affiliated Hospital of Nantong University. Depressive symptoms were assessed with the depression subscale of the Hospital Anxiety and Depression Scale (HAD-D). The quality of life was measured with the RAND 36-Item Health Survey (SF-36). And the independent risk factors of depressive symptoms were assessed by using a stepwise forward model of logistic regression analysis. *Results.* The mean age of the study subjects was 57.66 years (SD: 11.68). Approximately 21.4% of subjects reported depressive symptoms ($n = 45$). Forward stepwise logistic regression analysis showed that female gender ($P = 0.010$), hypertension ($P = 0.022$), Stage IV ($P = 0.003$), and Stage V ($P < 0.001$) were significant risk factors for depressive symptoms. The quality of life of individuals with HAD-D score <11 was significantly better compared with individuals with HAD-D score ≥ 11. *Conclusions.* These results indicate that clinicians should be aware that female patients with chronic kidney diseases with T2DM in their late stage with hypertension are at a marked increased risk of depressive symptoms. Providing optimal care for the psychological health of this population is vital.

1. Introduction

In China, high rates of unhealthy diets, physical inactivity, and obesity have led to an increase in the prevalence of type 2 diabetes mellitus (T2DM). Over 100 million Chinese people currently suffer from T2DM. T2DM is a chronic disease that in the long-term increases the microvascular degenerative complication of chronic kidney disease (CKD), which is a leading cause of end-stage renal disease (ESRD) [1]. ESRD requires either dialysis or transplantation, which is associated with cumulative hospital days and number of hospitalizations, contributing to excessive Medicare costs [2, 3]. CKD affects 20% to 40% of those who develop diabetes [4]. As the prevalence of T2DM in this country is increasing, the rate of CKD will also rise rapidly.

Depression is the third most important cause of disability in the world [5]. And having T2DM increases the risk of subsequent development or recurrence of depression. As an unwanted cotraveler of T2DM, depression can impinge on self-management ability and hinder patients adherence to treatment regime [6]. Furthermore, available studies have suggested that the presence of comorbid depression with diabetes is associated with poorer self-reported health status and higher adverse outcomes, including incident ESRD and mortality [7–13].

According to other documents, the prevalence of depression is observed in approximately 20% of patients with T2DM [14, 15]. Considering the high prevalence of CKD in patients with diabetes and that depression is rather common in patients with T2DM, the factors associated with depression effect on CKD in T2DM are a growing concern.

To our knowledge, the associated factors with depression were younger age, female gender, socioeconomic status, presence of cardiovascular disease, and health status [16, 17].

Furthermore, depression is associated with microalbuminuria in diabetes related CKD [18]. And low eGFR was regarded as a risk factor of depression in nondiabetic CKD [19, 20].

However, the factors associated with depressive symptoms among CKD in type 2 diabetic patients have not been examined in previous studies. Therefore we were specifically interested in the relationship between demographic, clinical variables, and depressive symptoms in CKD patients with T2DM.

The aim of this study was to assess the associations between depressive symptoms and kidney disease in an inpatient type 2 diabetic cohort and to learn about the warning signs of mood symptoms in these patients to provide early diagnosis and treatment.

2. Methods

2.1. Study Population and Recruitment. From October 2015 to April 2016, an observational cross-sectional study was conducted among the T2DM patients with CKD from the Second Affiliated Hospital of Nantong University. An eligible person fulfilled the 1999 World Health Organization (WHO) criteria for the diagnostic of T2DM or a hospital discharge diagnosis of T2DM. When abnormal estimated glomerular filtration rate (eGFR) or urinary albumin excretion rate (UAER) was presented in three months, T2DM patients would be categorized as having CKD. The severity of CKD in T2DM was categorized into five stages according to NKF-KDOQI Guidelines [21, 22] and the staging standard of CKD in type 1 diabetes [23]. Stage I: UAER < 10 μg/min and eGFR increased obviously, kidney size increased; Stage II: UAER < 20 μg/min or increased intermittently (such as exercise, stress state), eGFR increased slightly; Stage III: UAER 20~200 μg/min (last for 3 months), eGFR is normal or above normal; Stage IV: UAER > 200 μg/min, eGFR decreased; Stage V: end-stage renal failure, eGFR < 10 ml/min. eGFR was estimated from modified MDRD equations---c-aGFR4 [24–26]. Cases were excluded on the basis of the following criteria: (1) age < 18 or >80 years at the time of interview; (2) pregnant and lactating women; (3) hearing and cognitive impairment; (4) any unstable medical illness or mental disease; and (5) those who did not complete the questionnaire. Finally, 210 patients were eligible for investigation and entered the study.

In compliance with the Helsinki declaration, all subjects were told about the concept of the study and signed an informed consent prior to commencement of the study. Approval to conduct this study was obtained from the Ethics Committee of the Second Affiliated Hospital of Nantong University.

2.2. Instruments. Two scales, the Hospital Anxiety and Depression Scale (HAD) and the RAND 36-Item Health Survey (SF-36), were used to evaluate depressive symptoms and quality of life (QOL). The HAD [27], a reliable and valid self-report rating scale [28], includes 7 items for anxiety (HAD-A) and 7 for depression (HAD-D). The total score is the sum of the 14 items with a four-point scale on each item (range 0–3). For each subscale, the score is the sum of the respective seven items (ranging from 0 to 21). We defined the scores of HAD-D greater than 11 as depressive symptoms.

QOL were assessed with the SF-36, comprising 36 items organized into 8 scales: physical functioning (PF) (10 items), role physical (RP) (4 items), body pain (BP) (2 items), general health (GH) (5 items), energy/fatigue (VT) (4 items), social functioning (SF) (2 items), role emotional (RE) (3 items), and mental health (MH) (5 items). In addition to the above 8 aspects, SF-36 also contains another health indicator: reported health transition (HT) (1 item), used to evaluate the health status of the overall change in the past year. A Chinese version of the SF-36 scale has been validated by Wang et al. [29].

Each participant underwent an in-person interview of demographic data (age, gender, income, level of education, health coverage, etc.), diabetes characteristics (duration of diabetes, insulin therapy), and self-care behavior (clinic visit, blood glucose monitoring). Detailed clinical parameters (hypertension, HbA1c, plasma creatinine, and albuminuria) were abstracted from electronic medical record system.

2.3. Statistical Analysis. Statistical analyses were performed with SSPS 21.0. Descriptive statistics were calculated for all variables measured. Data were reported as mean (standard deviation [SD]) or median (interquartile range, IQR) or percentages. Significant differences were determined by using independent t-tests (continuous variables with normal distribution), Chi-Square tests (categorical variables), and Mann–Whitney tests (continuous variables with skewed distribution). A stepwise forward model of logistic regression was used to determine independent factors of depressive symptoms with odds ratios (ORs) and the corresponding 95% CIs. Statistical significance was set at $P < 0.05$ (two tailed).

3. Results

A total of 223 patients were invited to participate. Thirteen (5.8%) were excluded because of incomplete data. Among the 210 total subjects, 45 (21.4%) patients with T2DM had symptoms of depression. In Tables 1(a) and 1(b), for HAD-D scores ≥ 11 patients, significant associations were found with female gender ($P = 0.006$), yearly income ($P = 0.016$), clinic visit frequency ($P = 0.018$), blood glucose monitoring frequency ($P = 0.019$), and duration of diabetes ($P = 0.035$). The HAD-D scores ≥ 11 were higher in T2DM complicated with hypertension (27.8%) compared with T2DM without hypertension (13.7%) ($\chi^2 = 6.18$; $P = 0.013$). The rates of depressive symptoms among patients in Stage IV and V were higher than patients in I–III ($\chi^2 = 23.28$; $P < 0.001$). About forty percent of the patients who reported depressive symptoms were found with low eGFR ($\chi^2 = 15.42$; $P < 0.001$) and macroalbuminuria ($\chi^2 = 17.49$; $P < 0.001$). However, there were no significant differences in depressive symptoms by age ($P = 0.533$), employment status ($P = 0.290$), education ($P = 0.150$), marital status ($P = 0.093$), insurance ($P = 0.117$), insulin use ($P = 0.334$), or HbA1c ($P = 0.921$).

Table 1: (a) Comparison of demographic factors of T2DM-related CKD patients by depressive symptoms status. (b) Comparison of clinical factors of T2DM-related CKD patients by depressive symptoms status.

(a)

Variables	Overall sample	Depression status		χ^2/z	P value
		HAD-D score < 11	HAD-D score ≥ 11		
Total, *n* (%)	210	165 (78.6)	45 (21.4)		
Gender, *n* (%)				7.507	**0.006**
Male	108 (51.4)	93 (86.1)	15 (13.9)		
Female	102 (45.1)	72 (70.6)	30 (29.4)		
Age (year), mean (SD)	57.66 (11.68)	57.39 (11.57)	58.62 (12.14)	−0.624*	0.533
Employment status, *n* (%)				1.118	0.290
Working	102 (48.6)	77 (75.5)	25 (24.5)		
Not working	108 (51.4)	88 (81.5)	20 (18.5)		
Education, *n* (%)				3.791	0.150
Primary or below (0–6 years)	65 (31.0)	46 (76.7)	19 (29.2)		
Secondary (7–13 years)	122 (58.1)	99 (79.2)	23 (18.9)		
Tertiary (>13 years)	23 (11.0)	20 (80.0)	3 (13.0)		
Marital status (married), *n* (%)	189 (90.0)	152 (80.4)	37 (19.6)	2.828	0.093
Yearly income (RMB), *n* (%)				8.229	**0.016**
<15000	42 (20.0)	28 (66.7)	14 (33.3)		
15000~33000	75 (35.7)	56 (74.7)	19 (25.3)		
>33000	93 (44.3)	81 (87.1)	12 (12.9)		
Insurance, *n* (%)				2.452	0.117
Rural medicare cooperative	64 (30.5)	46 (71.9)	18 (28.1)		
Medical insurance	146 (69.5)	119 (81.5)	27 (18.5)		

Bold values indicate significant results ($P < 0.05$).
*Independent t-tests.

(b)

Variables	Overall sample	Depression Status		χ^2/z	P value
		HAD-D score < 11	HAD-D score ≥ 11		
clinic visit frequency (times/year), median (IQR)	0.5 (1.0)	0.0 (1.0)	1.00 (2.0)	−2.356	**0.018**
blood glucose testing (times/month), median (IQR)	1.0 (4.0)	1.0 (4.0)	2.0 (3.0)	−2.336	**0.019**
Duration of diabetes (years), median (IQR)	7.0 (12.0)	6.0 (12.92)	8.0 (10.5)	−2.111*	**0.035**
Insulin use (yes), *n* (%)	116 (55.2)	94 (81.0)	22 (19.0)	0.934	0.334
Hypertension (yes), *n* (%)	115 (54.8)	83 (72.2)	32 (27.8)	6.180	**0.013**
HbA1c				0.010	0.921
≤9	78 (37.1)	61 (78.2)	17 (21.8)		
>9	132 (62.9)	104 (78.8)	28 (21.2)		
eGFR (mL/min/1.73 m^2), *n* (%)				15.420	<**0.001**
≥90	75 (35.7)	68 (90.7)	7 (9.3)		
60–89	105 (50.0)	80 (76.2)	25 (23.8)		
<60	30 (14.3)	17 (56.7)	13 (43.3)		
Albuminuria (mg/L), *n* (%)				17.485	<**0.001**
Normal Albuminuria (<20)	109 (51.9)	96 (88.1)	13 (11.9)		
Microalbuminuria (20~200)	59 (28.1)	45 (76.3)	14 (23.7)		
Macroalbuminuria (>200)	42 (20.0)	24 (57.1)	18 (42.9)		

(b) Continued.

Variables	Overall sample	Depression Status		χ^2/z	P value
		HAD-D score < 11	HAD-D score ≥ 11		
Severity of DKD, n (%)				23.283	<**0.001**
Stage I	74 (35.2)	68 (91.9)	6 (8.1)		
Stage II	41 (19.5)	31 (75.6)	10 (24.4)		
Stage III	55 (26.2)	44 (80.0)	11 (20.0)		
Stage IV	24 (11.4)	15 (62.5)	9 (37.5)		
Stage V	16 (7.6)	7 (43.8)	9 (56.3)		

Bold values indicate significant results ($P < 0.05$).
*Mann-Whitney test.

Table 2: Logistic regression model of T2DM-related CKD patients on depressive symptoms.

Variables	B	SE	Wald	OR	exp (B) 95% CI		P value
					Lower bound	Upper bound	
Hypertension							
Without hypertension	—	—	—	REF	—	—	—
With hypertension	0.902	0.395	5.208	2.465	1.136	5.350	**0.022**
Severity of T2DM-related CKD			16.779				**0.002**
Stage I	—	—	—	REF	—	—	—
Stage II	1.056	0.576	3.357	2.875	0.929	8.899	0.067
Stage III	0.837	0.558	2.252	2.309	0.774	6.886	0.133
Stage IV	1.877	0.622	9.095	6.533	1.929	22.122	**0.003**
Stage V	2.464	0.682	13.056	11.755	3.088	44.744	**0.000**
Gender							
Female	0.993	0.387	6.586	2.699	1.264	5.760	**0.010**
Male	—	—	—	REF	—	—	—

REF: reference group.
Bold values indicate significant results ($P < 0.05$).

Forward stepwise logistic regression analysis was used to identify a model to predict T2DM-related CKD patient who would have depressive symptoms. The results showed that variables of female gender, severity of T2DM-related CKD, and complicated hypertension were significant risk factors for depressive symptoms (Table 2). This model had a good fit under the Hosmer-Lemeshow goodness-of-fit test ($R^2 = 0.233$, $\chi^2 = 7.568$, $P = 0.477$), and accuracy of the model was 78.5%.

T2DM patients with hypertension were 2.5 times more likely to experience depressive symptoms than T2DM patients without hypertension ($P = 0.022$). The likelihood of depressive symptoms in Stage IV was 6.5 times more than that in stage I, and Stage V was 11.8 times. Female patients were 2.6 times more likely to report depressive symptoms than male patients.

Finally, the subjects were divided into two groups according to the HAD-D score. An analysis showed that all the dimension scores of SF-36 in individuals with HAD-D score < 11 were significantly higher compared with individuals with HAD-D score ≥ 11 ($P < 0.05$). The results are shown in Table 3.

4. Discussion

We found that almost 21.4% of subjects had depressive symptoms (HAD-D score ≥ 11). And the depressive symptoms were negatively associated with patients' quality of life. Depressive symptoms are becoming a serious problem.

We also found the differences between T2DM patients with and without depressive symptoms were yearly income, clinic visit frequency, blood glucose testing frequency, diabetic duration, eGFR, and albuminuria by univariate analysis. The lower compliance to the self-care measures of clinic visit and blood glucose testing will lead to a poor glycemic control which can contribute to the occurrence of diabetic complications [30, 31]. Besides, an increased duration of T2DM contributes significantly to an increase in the risk of diabetic complications. Low eGFR and macroalbuminuria revealed severe renal impairment which could require dialysis or kidney transplantation. These adverse consequences will lead to the increased economic burden of healthcare costs for lower income group. Due to economic instability and increased healthcare expenditures for complications and adherence to treatment, these patients are more susceptible to experiencing psychological diseases [32].

Table 3: Comparison between the HAD-D score ≥ 11 group and HAD-D score < 11 group in SF-36 domain score.

QOL assessments (SF-36 domains), mean (SD)	Overall sample	HAD-D score < 11	HAD-D score ≥ 11	Z	P value
PF	85.52 (23.62)	88.86 (19.83)	59.55 (33.45)	−4.685	**<0.001**
RP	62.82 (44.42)	67.40 (42.71)	27.27 (42.19)	−3.649	**<0.001**
BP	85.81 (22.63)	87.30 (22.19)	74.27 (23.25)	−2.929	**0.003**
GH	57.28 (24.33)	60.47 (23.23)	32.50 (17.85)	−4.919	**<0.001**
VT	65.03 (19.01)	66.96 (18.36)	50.00 (17.53)	−3.933	**<0.001**
SF	91.26 (18.76)	94.23 (14.17)	68.18 (31.04)	−5.033	**<0.001**
RE	89.12 (30.66)	93.18 (25.29)	57.58 (47.34)	−4.969	**<0.001**
MH	82.32 (11.72)	84.40 (8.38)	66.18 (19.47)	−4.714	**<0.001**
HT	39.38 (18.33)	40.94 (18.14)	27.27 (15.25)	−3.185	**0.001**
PCS	72.82 (21.70)	75.96 (19.83)	48.40 (20.47)	−5.056	**<0.001**
MCS	81.90 (15.28)	84.66 (11.77)	60.48 (21.71)	−5.994	**<0.001**
Total score	71.54 (13.20)	73.93 (11.00)	53.01 (14.36)	−5.790	**<0.001**

Bold values indicate significant results ($P < 0.05$).
PCS: the physical component summary.
MCS: the mental component summary.

However, age, being unmarried, low monthly income, low educational level, unemployment, and HbA1c associated with depressive symptoms occurrence in T2DM patients have been proved in previous studies [30, 33–36]. These associations were not revealed in our 210 patients. We expect that this is due to a relatively low power, caused by the relatively low number of cases experiencing depressive symptoms. This is a main limitation of our study. Besides, variations in study design and participants' demographic characteristics might also be the reasons for the discrepancy.

In the final stepwise logistic regression we found that female gender was an independent risk factor for depressive symptoms, which was in line with previous studies [37, 38]. Sun et al. [39] found an association between being woman and depressive symptoms. This sex difference could be due to lower physical activity lifestyle and being more emotional than men.

Besides being female, hypertension was also an independent risk factor for depressive symptoms among T2DM-related CKD patients by stepwise logistic regression. Based on the previous studies [40, 41], hypertension has been demonstrated to be an independent risk factor of depressive symptoms among patients with T2DM due to increased risk of serious cardiovascular disease complications, reduced quality of life, and poor prognosis.

Additionally, the stepwise logistic regression analysis also indicated that the T2DM-related CKD progression was significantly associated with depressive symptoms. CKD with type 2 diabetic patients were reported to experience symptoms like inability to sleep, depression, and lack of energy when they perceived gradual worsening of their symptoms. As the disease progresses, the decline of the renal function eventually resulted in the outcomes of dialysis. Above feelings will be aggravated gradually [42]. Similarly, our results also showed that the prevalence of depressive symptoms was gradually increased along with the progress of disease. In stages I–V, depression was 8.1%, 24.4%, 20.0%, 37.5%, and 56.3%, respectively. About sixty percent of ESRD symptoms would be complicated by depressive symptoms. Young et al. [4] suggested further testing of targeted depression interventions should be considered in this population, for the reason that major depression at baseline was associated with a 3-fold greater risk of mortality among Stage V CKD diabetic patients.

At the end, there are several limitations in our research that should be addressed. First, our sample was selected from one hospital in China for convenience. The study cannot be considered as representative of the T2DM-related CKD population in China. Second, we used self-reported and closed-ended questionnaires. Third, the number of complications and treatments of ESRD were not considered. Fourth, the sample size in this study is relatively smaller than some other studies.

In conclusion, T2DM-related CKD patients showed a higher predisposition to depression. Medical staff should pay more attention to the psychological issues when T2DM patients were involved in the treatment of CKD, especially for female patients in late stage complicated with hypertension.

Competing Interests

The authors declare that they have no competing financial interests.

Authors' Contributions

Weiqun Weng and Xueqin Wang designed the study. Xu Wang and Biyu Shen acquired and analyzed the data. Xun Zhuang interpreted the data. Xu Wang wrote the paper. All authors reviewed the manuscript.

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Research Article

Renal Protective Effect of DPP-4 Inhibitors in Type 2 Diabetes Mellitus Patients: A Cohort Study

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Aims. Dipeptidyl-peptidase IV inhibitors (DPP-4i) are among the most popular oral antidiabetic agents. However, the effects of DPP-4i on diabetic nephropathy are not well-established. The aim of this study was to determine the renoprotective effects of DPP-4i, using albuminuria and glomerular filtration rate (GFR) as indicators, in type 2 diabetes mellitus (T2DM) patients. *Methods*. This retrospective observational cohort study used the clinical database of a tertiary hospital. The changes of urine albumin/creatinine ratio (UACR), estimated GFR (eGFR), and metabolic parameters after treatment were compared with the changes of those parameters before treatment using paired Student's t-test. *Results*. The mean UACR in the entire study population decreased to approximately 45 mg/g 1 year after DPP-4i treatment, while it was increased approximately 39 mg/g 1 year before DPP-4i treatment ($p < 0.05$). Patients with macroalbuminuria showed a significant reduction in albumin levels after DPP-4i treatment ($p < 0.05$); however, patients with microalbuminuria and normoalbuminuria did not show improvements in albuminuria levels after treatment. Although eGFR was not changed 1 year after DPP-4i treatment, reductions in eGFR were slowed in patients with microalbuminuria and reversed in the macroalbuminuria or normoalbuminuria groups, 4 years after treatment. *Conclusions*. Administration of DPP-4i reduces urine albumin excretion and mitigates reduction of eGFR in T2DM patients.

1. Introduction

Diabetic nephropathy is one of the most important complications of diabetes, being strongly associated with increased overall mortality, cardiovascular mortality, cardiovascular events, and end-stage renal disease [1, 2]. The major and earliest clinical manifestation of diabetic nephropathy is albuminuria. Albuminuria is a principal marker of kidney damage, which is caused by glomerular leakage. Generally, albuminuria is used as a marker of diabetic nephropathy; many studies have suggested that albuminuria is correlated with the progression of diabetic nephropathy, cardiovascular mortality, and all-cause mortality [3–6]. In addition, reduction of albuminuria with drugs is associated with renal protection [7–9]. However, some studies have suggested that albuminuria is not an appropriate therapeutic target for diabetic nephropathy [10]. Indeed, both loop and thiazide diuretics resulted in a reduction of albuminuria but did not improve renal outcome [11, 12]. For this reason, albuminuria and glomerular filtration rate (GFR) should be considered together as surrogate markers for diabetic nephropathy.

Dipeptidyl-peptidase IV inhibitors (DPP-4i) are among the most popular and effective oral antidiabetic agents. They have many advantages, including high glucose-lowering potency, low risk of hypoglycemia, no association with weight gain, and being tolerable in chronic renal failure patients. However, their efficacy for preventing diabetic complications, especially diabetic nephropathy, is not well-established. Physiologically, dipeptidyl-peptidase IV (DPP-4) acts on nephrons to exert various functions [13, 14], and some preclinical studies have suggested that DPP-4i exerts renoprotective effects [15–17]. However, clinical evidence

regarding the renal protective effects of DPP-4i therapy is limited.

The majority of clinical studies investigating the renoprotective effects of DPP-4i have only focused on evaluating their albuminuria-lowering ability [18, 19]. Although several studies have been proposed on this topic, it is still unclear that DPP-4i slows the deterioration of GFR in diabetic nephropathy [20, 21]. Moreover, almost all of these studies only contained short-term follow-up data [18–22] and included only one DPP-4i drug; thus, they were unable to evaluate effects according to drug class.

The current study aimed to determine the renoprotective effects of DPP-4i, using albuminuria and GFR as indicators, in type 2 diabetes mellitus (T2DM) patients.

2. Methods

2.1. Study Design and Subjects. A retrospective observational cohort study was conducted using the clinical database of Ajou University Hospital, which is a South Korean tertiary hospital with 1,108 beds. The study protocol was approved by the Institutional Review Board of Ajou University Hospital.

The inclusion criteria for study participants were as follows: (1) aged ≥ 19 years with T2DM identified by the International Classification of Disease, Tenth Revision code E11; (2) prescribed DPP-4i from March 1, 2010, to February 28, 2014; and (3) data on urine albumin and creatinine levels at baseline, 1 year prior to starting DPP-4i, and 1 year after DPP-4i treatment initiation.

Exclusion criteria were as follows: glycosylated hemoglobin (HbA1c) ≤ 6.5% (48 mmol/mol) or >10% (86 mmol/mol); baseline estimated GFR (eGFR) ≤ 15 mL/min/1.73 m^2; currently undergoing dialysis; body mass index (BMI) > 40 kg/m^2; treatment with insulin; treatment with steroids for >7 days; and patients with dual blockade of the renin-angiotensin system (RAS).

A total of 414 patients were included in this cohort. The patients were divided into three groups according to their baseline urine albumin creatinine ratio (UACR): (1) a macroalbuminuria group (UACR ≥ 300 mg/g, $n = 38$), (2) a microalbuminuria group (30 mg/g ≤ UACR < 300 mg/g, $n = 116$), and (3) a normoalbuminuria group (UACR < 30 mg/g, $n = 260$). Additionally, we performed a long-term efficacy analysis to evaluate the effects of DPP-4i on the eGFR. Patients in the study cohort who had been prescribed DPP-4i continuously for more than 4 years and whose serum creatinine levels were measured at baseline, 4 years before and after the first prescription of DPP-4i, were included in the long-term efficacy analysis.

2.2. Data Extraction. The first prescription date of DPP-4i was defined as the index date, and the first prescribed DPP-4i was classified as the treatment drug in patients who were prescribed more than one DPP-4i. Cessation of DPP-4i therapy was designated as the date of changing to another antidiabetic drug, a drug prescription gap of more than 30 days, or the study end date (May 31, 2015). Drug adherence was measured using the proportion of days covered (PDC, the days of taking the medicine divided by a whole follow-up duration). PDC ≥ 0.80 was considered to indicate drug adherence and patients with PDC < 0.80 were removed from the analyses.

Demographic characteristics, including age and gender, were extracted from index data. Blood pressure, height, weight, diabetes mellitus (DM) duration, and baseline laboratory tests—including HbA1c, lipid profile, serum creatinine, urine creatinine, and urine albumin—were collected (i.e., the most recent values measured within 90-day range prior to the index date). Values for these parameters before and after treatment were also extracted using the same method. UACR was calculated using urine albumin and creatinine levels from an untimed spot urine collection. eGFR was measured using the Modification of Diet in Renal Disease Study Equation [23]:

$$\text{eGFR} = \Big(186 \times (\text{serum creatinine})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female})\Big). \quad (1)$$

2.3. Statistical Analysis. All analyses were performed using R software (ver. 3.2.3; R Development Core Team, Vienna, Austria). Data are expressed as means ± standard deviation. A self-controlled design, in which comparisons are made within individuals, was used to estimate the renoprotective effect of DPP-4i. Using this method, all time-invariant confounders (e.g., sex, smoking, ethnicity, albuminuria status, other underlying diseases, and coadministrated drugs) were eliminated, and time-constant covariates (e.g., age, eGFR deterioration due to DM, and DM duration) were properly adjusted for. The paired Student's t-test was used to evaluate statistical differences between all parameters before and after DPP-4i treatment. Multiple linear regression analysis was performed to evaluate the effects of covariates on albuminuria reduction.

3. Results

3.1. Patients Characteristics. A total of 414 patients with T2DM satisfied the eligibility criteria of this study. The mean age of the included patients was 59.2 ± 11.5 years and the mean duration of DM was 11.0 ± 7.4 years. The mean BMI and HbA1c were 25.2 ± 3.6 kg/m^2 and 8.6 ± 1.5%, respectively. Metformin and sulfonylurea were prescribed in 74.9% and 69.8% of patients, respectively, while 56.8% of patients were prescribed RAS inhibitors and 59.2% of patients were prescribed statins (Table 1).

3.2. Changes in UACR and Metabolic Parameters 1 Year prior to and 1 Year after DPP-4i Treatment. The mean UACR in all patients increased approximately 39 mg/g from 1 year before DPP-4i treatment to the point of DPP-4i treatment initiation, while it was decreased approximately 45 mg/g 1 year after initiation of DPP-4i treatment ($p < 0.05$). Patients with macroalbuminuria (≥300 mg/g) showed significant reductions in albuminuria (Figure 1, $p < 0.05$); however, patients

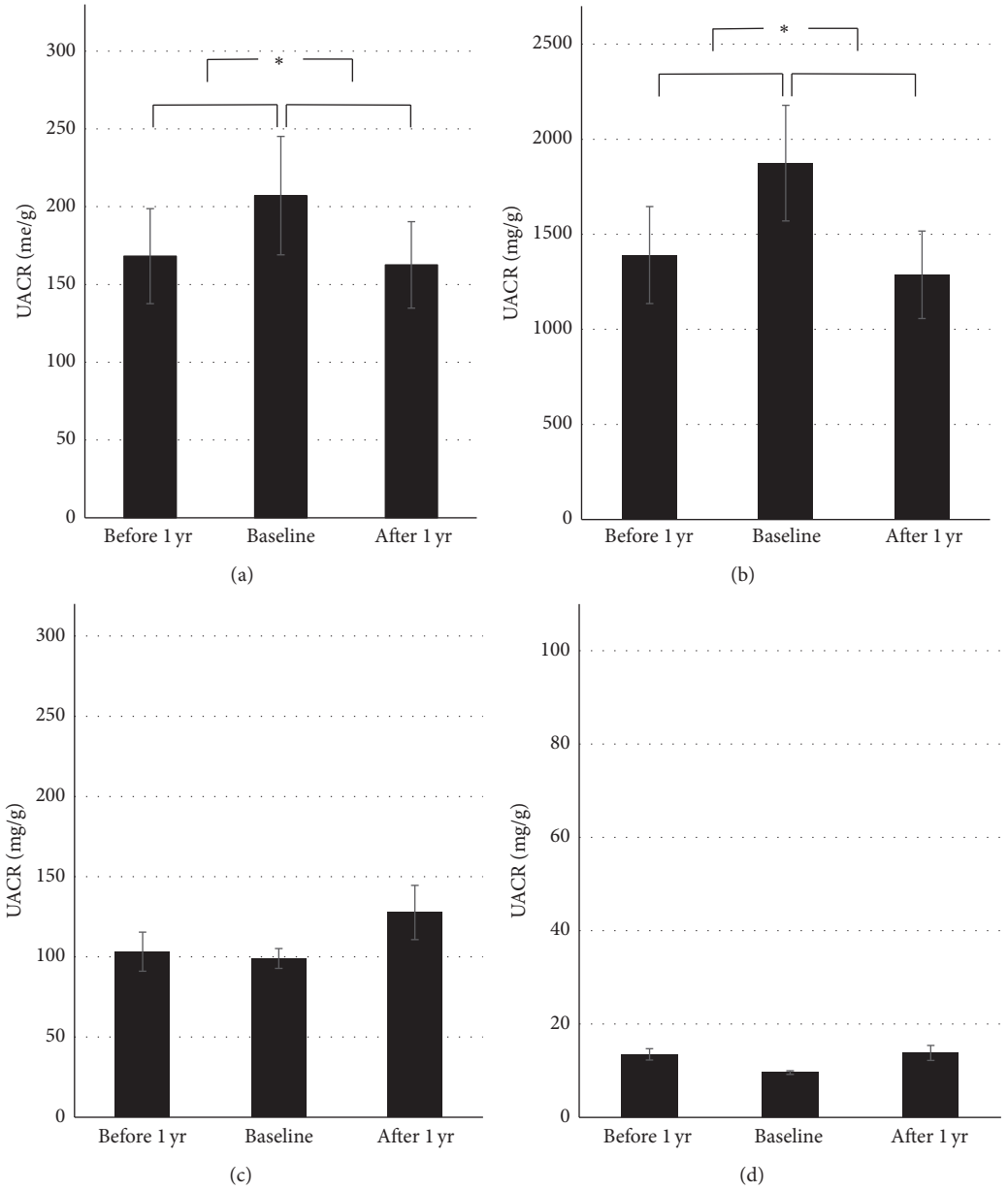

Figure 1: Changes in urine albumin/creatinine ratio 1 year before and 1 year after DPP-4i treatment initiation. Changes in urine albumin/creatinine ratio in all patients (a) and in patients with macroalbuminuria (b), microalbuminuria (c), and normoalbuminuria (d). (Data are presented as means with standard errors.) DPP-4i: dipeptidyl-peptidase IV inhibitor; UACR: urine albumin/creatinine ratio. $^{*}p$ value < 0.05.

with microalbuminuria and normoalbuminuria showed no significant changes.

The mean HbA1c improved from 8.6% (70 mmol/mol) to 7.8% (62 mmol/mol) ($p < 0.01$), and the mean low-density lipoprotein- (LDL-) cholesterol level decreased from 89.8 ± 39.5 mg/dL to 84.4 ± 33.1 mg/dL ($p < 0.05$). However, eGFR was not changed 1 year after DPP-4i treatment compared with 1 year before DPP-4i treatment (Table 2).

3.3. Estimating the Effect of Covariates on Albuminuria Reduction. To estimate the effect of covariates on albuminuria reduction, multiple linear regression analysis was performed. Although a reduction of HbA1c was shown using the paired Student's t-test, no significant decrease was seen on multiple linear regression analysis. Moreover, sex, age, and systolic blood pressure did not explain the changes seen in UACR on multiple linear regression analysis (Table 3).

3.4. Changes in eGFR 4 Years prior to and 4 Years after DPP-4i Treatment. To verify the long-term effects of DPP-4i on eGFR, the change in eGFR from a point of treatment to 4 years before DPP-4i treatment and 4 years after treatment

Table 1: Patient baseline characteristics ($N = 414$).

Characteristics	Results
Age (years)	59.2 ± 11.5
Sex (*n*, male/female)	224/190
Body mass index (kg/m^2)	25.2 ± 3.6
DM duration (years)	11.0 ± 7.4
Systolic blood pressure (mmHg)	125.4 ± 17.2
Diastolic blood pressure (mmHg)	73.3 ± 10.6
HbA1c (%)	8.6 ± 1.5
LDL-cholesterol (mg/dL)	89.8 ± 39.5
HDL-cholesterol (mg/dL)	46.4 ± 12.0
Triglycerides (mg/dL)	168.0 ± 35.5
eGFR (mL/min/1.73 m^2)	68.3 ± 17.6
Antidiabetic drugs (%)	
Metformin	74.9
Sulfonylurea	69.8
Thiazolidinedione	3.6
Alpha-glucosidase inhibitor	0.2
RAS inhibitor (%)	56.8
Statin (%)	59.2

Data are presented as means ± standard deviation or frequencies.
eGFR: estimated glomerular filtration rate; HbA1c: glycosylated hemoglobin; HDL: high density lipoprotein; LDL: low density lipoprotein; RAS: renin-angiotensin system.

was compared in patients who were prescribed DPP-4i for more than 4 years. A total of 78 patients were included in the analysis (characteristics of those patients were present in Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1423191). The mean change in eGFR 4 years before treatment from baseline was −22.4, −9.1, and −8.3 mL/min/1.73 m^2 in the macroalbuminuria, microalbuminuria, and normoalbuminuria groups, respectively (Figure 2). However, 4 years after DPP-4i treatment initiation, the eGFR increased in the macroalbuminuria group from 54.3 to 58.5 mL/min/1.73 m^2 and in the normoalbuminuria group from 70.3 to 77.5 mL/min/1.73 m^2. In each group, paired Student's t-test on the eGFR change from a point of treatment to 4 years before DPP-4i treatment and 4 years after treatment was statistically significant ($p < 0.01$ for all groups).

3.5. Subgroup Analysis for Sex, Age, Obesity, Chronic Kidney Disease Stage, and Drug Coadministration. A subgroup analysis was performed to determine which subgroup was associated with UACR changes and what factors were associated with the albuminuria-lowering effect of DPP-4i. Albuminuria significantly decreased in patients < 65 years old of both genders ($p < 0.05$). However, no significant differences in albuminuria were found when patients were divided according to their chronic kidney disease stage. Patients who were prescribed metformin, statins, and RAS inhibitors showed improvement in albuminuria ($p < 0.05$). Vildagliptin, sitagliptin, saxagliptin, and linagliptin decreased albuminuria without statistical significance (Table 4).

4. Discussion

This retrospective cohort study suggests that DPP-4i could reduce UACR, especially in T2DM patients with macroalbuminuria. Interestingly, DPP-4i reduced albuminuria in patients who were coadministered metformin or statins. Furthermore, DPP-4i could preserve eGFR in patients with T2DM, regardless of their baseline UACR.

Some mechanisms have been suggested to underlie the renoprotective effects of DPP-4i in previous studies. DPP-4 shows the highest expression in the kidneys among all organs and is mainly expressed in the kidney proximal tubule in healthy humans [24]. However, in DM patients, DPP-4 is also present in the renal glomerulus [25]. DPP-4 inhibition by DPP-4i was shown to reduce kidney injury in rat models of diabetes [16, 17]. One suggested mechanism underlying this effect is that DPP-4 inhibition upregulates renal cyclic adenosine monophosphate (cAMP) production by elevating circulatory stromal cell-derived factor-1a [26]. Increased cAMP has antioxidative effects and reduces reactive oxygen species, which are considered a major cause of diabetic nephropathy. Another suggested mechanism is that DPP-4i elevates active glucagon-like peptide-1, which is known to upregulate cAMP and reduce oxidative stress [27].

In our study, eGFR was increased in patients with macroalbuminuria or normoalbuminuria after taking DPP-4i ($p < 0.01$), while the eGFR reduction rate in patients with microalbuminuria was slower during the 4 years after DPP-4i initiation relative to prior to treatment initiation ($p < 0.05$). To our knowledge, this is the first study to show that DPP-4i alleviates eGFR decline. Previous studies have shown that eGFR was not improved after 3–6 months of DPP-4i treatment [20, 21]. Similarly, eGFR was not different 1 year after DPP-4i treatment but was improved after 4 years of treatment in our study. Thus, long-term DPP-4i treatment may mitigate the decline in eGFR associated with diabetic nephropathy or even improve it.

Urine albumin excretion was decreased after DPP-4i treatment in the macroalbuminuria group ($p < 0.005$) but not in the microalbuminuria and normoalbuminuria groups. This result is not consistent with previous studies, which suggested that DPP-4i had an albuminuria-lowering effect in patients with microalbuminuria [18, 21]. However, the follow-up periods of the above-mentioned studies were shorter than that of our cohort (3–6 months) and their patients were prescribed only sitagliptin. Moreover, although HbA1c was decreased after DPP-4i administration, the effect of HbA1c on lowering albuminuria was not significant in multiple linear regression. The change in albuminuria was not influenced by sex, age, DM duration, eGFR, or systolic blood pressure at baseline.

In the subgroup analysis pertaining to coadministration of other drugs, urine albumin excretion was decreased in patients who were given metformin, statins, and RAS inhibitors. The renoprotective efficacy of metformin in T2DM patients remains controversial. Some studies insist that metformin lowers urine albumin excretion and has renoprotective effects [28, 29]; however, other studies have shown that metformin did not exert these beneficial and

Table 2: Changes in UACR, HbA1c, eGFR, and lipid profiles 1 year before and 1 year after DPP-4i treatment initiation.

	Changes during 1 year before treatment	Changes during 1 year after treatment	p value[‡]
UACR (mg/g)	40.8 ± 307.8	−44.5 ± 351.9	<0.05
HbA1c (%)	0.4 ± 1.1	−0.8 ± 1.5	<0.01
Systolic blood pressure (mmHg)	−1.2 ± 20.5	1.9 ± 20.7	0.13
Diastolic blood pressure (mmHg)	−0.3 ± 12.8	1.4 ± 12.7	0.37
LDL-cholesterol (mg/dL)	−1.2 ± 26.32	−3.5 ± 30.2	<0.05
HDL-cholesterol (mg/dL)	−0.3 ± 8.7	−0.9 ± 8.4	0.30
eGFR (mL/min/1.73 m^2)	−0.7 ± 8.7	1.2 ± 11.3	0.69

Data are presented as means ± standard deviation.
[‡]The paired Student's t-test was performed to evaluate changes in each parameter from baseline to 1 year before DPP-4i treatment and 1 year after treatment initiation.
DPP-4i: dipeptidyl-peptidase IV inhibitor; eGFR: estimated glomerular filtration rate; HbA1c: glycosylated hemoglobin; HDL: high density lipoprotein; LDL: low density lipoprotein; UACR: urine albumin/creatinine ratio.

Table 3: Multiple linear regression analysis for predictors of change of UACR.

	β	p value
Age	−0.002	0.78
Sex (male)	0.263	0.06
Duration of diabetes	0.010	0.18
BMI	0.003	0.77
Systolic blood pressure	0.006	0.12
ΔHbA1c	0.035	0.36
ΔLDL-cholesterol	0.001	0.63
eGFR	0.001	0.98

BMI: body mass index; eGFR: estimated glomerular filtration rate; HbA1c: glycosylated hemoglobin; LDL: low-density lipoprotein.

protective effects [30, 31]. In our analysis, T2DM patients who were prescribed DPP-4i with metformin showed an improvement of albuminuria ($p < 0.05$). The combination of metformin and DPP-4i may have a synergistic renoprotective effect. However, a well-designed study with adequate power is warranted to verify this protective effect. Usually, statins disturb the uptake of plasma proteins in the glomerular tubule, which can result in albuminuria in some patients [32, 33]. In our analysis, administration of DPP-4i in patients also taking statins resulted in improvements in urine albumin excretion. Physiologically, DPP-4 and the glucagon-like peptide-1 (which is the substrate of DPP4) receptor are localized in the renal tubule. DPP-4i may prevent statin-induced proteinuria via unknown mechanisms, as well as decrease albuminuria caused by DM. However, our study was not specifically designed to evaluate any statin-induced proteinuria-lowering effect. Thus, large prospective cohort studies are needed to assess the synergistic effect of DPP-4i and statins on reduction of albuminuria.

An important strength of our study was that the cohort contained long-term treatment data. Previous studies investigating the renoprotective effects of DPP-4i used only short-term data, and they showed only albuminuria-lowering effects [18–21, 34]. Because our cohort contained patients' data 4 years prior to and 4 years following treatment initiation, we were able to demonstrate declines in eGFR being reduced and even reversed. A second strength of our study lies in the fact that we included five DPP-4i classes: sitagliptin, linagliptin, saxagliptin, vildagliptin, and gemigliptin. Although there were no significant differences between these drugs, the majority were associated with a reduction of albuminuria. It is possible that various DPP-4i classes exert different albuminuria-lowering effects in T2DM patients.

There were some limitations to our study. First, this study used a self-controlled design, as there was no control group. In this design, patient data prior to DPP-4i treatment were compared with data after DPP-4i treatment to estimate the effect of DPP-4i. Although there are some weaknesses in this design, time-invariant confounders and time-constant covariates were properly adjusted for using each patient's own data. Secondly, UACR was calculated from an untimed spot urine collection. A timed urine collection would better confirm albuminuria, as there are some diurnal variations and other conditions that affect creatinine excretion. However, timed urine collection is difficult under clinical circumstances because it is inconvenient. Unlike our retrospective study, a prospective cohort study,

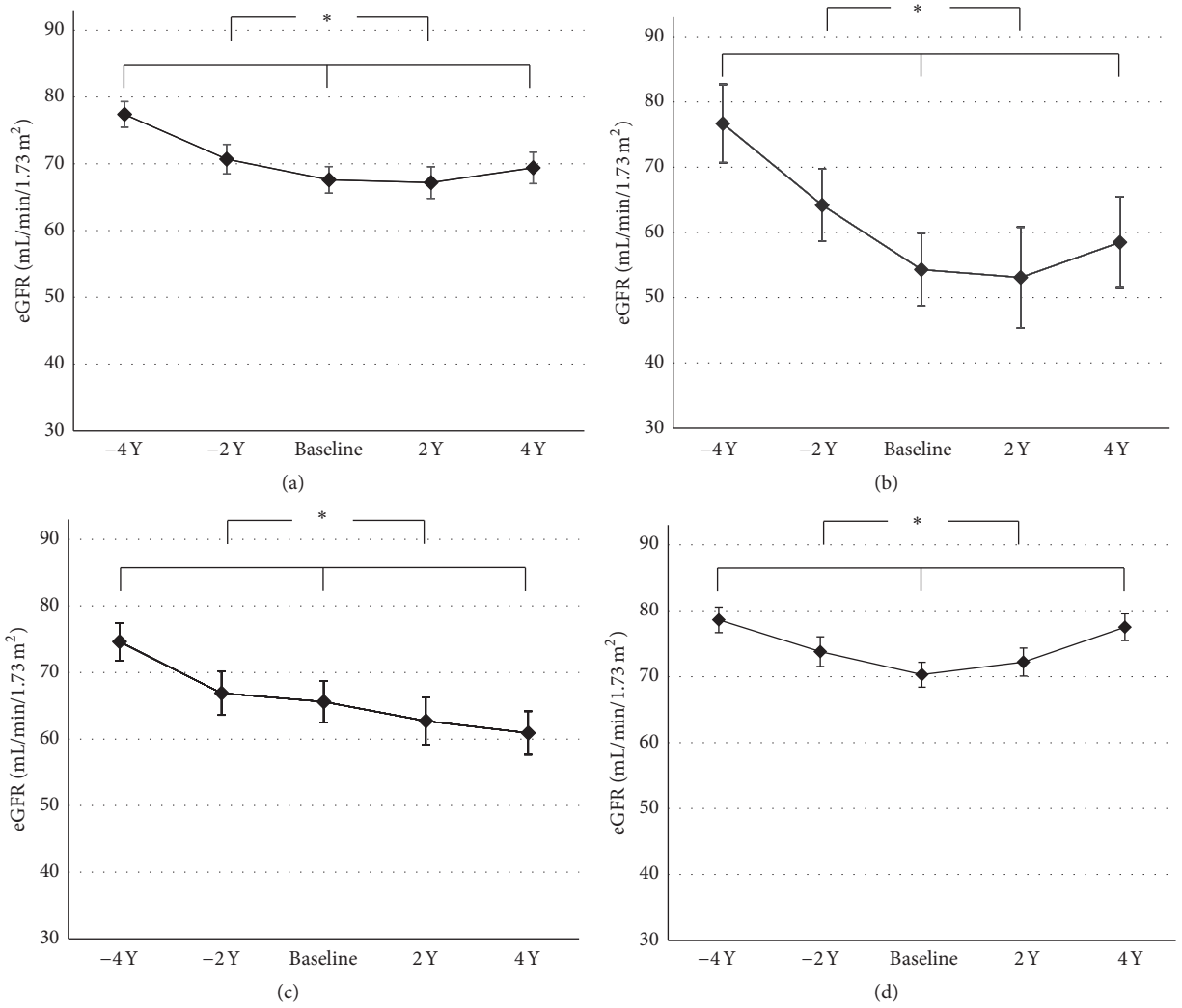

Figure 2: Changes in eGFR 4 years before and 4 years after DPP-4i treatment initiation. Changes in eGFR in all patients (a) and in patients with macroalbuminuria (b), microalbuminuria (c), and normoalbuminuria (d). Baseline values are the means with standard errors. DPP-4i: dipeptidyl-peptidase IV inhibitor; UACR: urine albumin/creatinine ratio; −4 Y: 4 years before DPP-4i treatment initiation; −2 Y: 2 years before DPP-4i treatment initiation; 2 Y: 2 years after DPP-4i treatment initiation; 4 Y: 4 years after DPP-4i treatment initiation. * p value < 0.01.

like MARLINA-T2D study, will be able to circumvent such disadvantages [19].

In conclusion, the present study demonstrated that DPP-4i treatment could ameliorate diabetic nephropathy, by reducing urine albumin excretion and mitigating the reduction of eGFR in T2DM patients.

Competing Interests

The authors declare no competing interests.

Authors' Contributions

Young-Gun Kim and Jung Hyun Byun contributed equally to this work as co-first authors. Young-Gun Kim designed the study, analyzed data, and wrote the manuscript. JungHyun Byun and Dukyong Yoon collected the data and analyzed data. Ja Young Jeon, Seung Jin Han, Dae Jung Kim, and Kwan-Woo Lee contributed to the discussion and reviewed and edited the manuscript. Rae Woong Park and Hae Jin Kim designed the study and wrote the manuscript.

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Table 4: Subgroup analysis for sex, age, obesity, chronic kidney disease stage, and drug coadministration.

	N	UACR 1 year before treatment	Baseline UACR	UACR 1 year after treatment	UACR change during 1 year before treatment	UACR change during 1 year after treatment	p value[‡]
Sex							
Male	224	127.2 ± 401.4	168.5 ± 541.8	135.6 ± 398.1	41.3 ± 227.3	−32.9 ± 282.6	<0.05
Female	190	212.4 ± 802.2	252.5 ± 979.7	194.3 ± 714.3	40.1 ± 380.4	−58.2 ± 419.5	<0.05
Age							
≥65 years	133	153.2 ± 384.0	166.0 ± 551.2	151.7 ± 484.5	12.8 ± 231.3	−14.3 ± 273.4	0.66
<65 years	281	171.0 ± 715.1	226.5 ± 860.1	167.7 ± 600.9	55.5 ± 340.9	−58.8 ± 382.8	<0.05
Obesity							
Obese	151	162.0 ± 654.5	232.5 ± 869.5	172.3 ± 585.1	70.5 ± 437.2	−60.2 ± 433.2	0.24
Nonobese	159	206.0 ± 770.5	256.8 ± 887.4	220.6 ± 691.7	50.8 ± 267.1	−36.2 ± 348.6	0.10
CKD							
eGFR ≥ 90	41	131.2 ± 231.1	126.6 ± 491.6	62.2 ± 158.0	−4.6 ± 67.3	−64.4 ± 419.3	0.92
90 > eGFR ≥ 60	229	91.1 ± 496.3	104.6 ± 524.7	81.6 ± 368.7	13.5 ± 149.0	−23.0 ± 229.9	0.29
60 > eGFR ≥ 30	115	306.2 ± 652.8	338.2 ± 864.5	286.7 ± 716.5	32 ± 261.5	−51.5 ± 383.8	0.11
30 > eGFR ≥ 15	13	861.8 ± 1631.2	917.6 ± 2446.1	886.9 ± 1599.0	55.8 ± 1181.9	−30.7 ± 1074.0	0.27
Metformin							
Yes	310	156.0 ± 573.2	187.5 ± 678.2	137.0 ± 508.3	21.9 ± 193.7	−50.5 ± 319.8	<0.05
No	104	174.6 ± 737.5	265.3 ± 1009.5	238.7 ± 707.0	90.7 ± 495.1	−26.6 ± 435.0	0.25
Sulfonylurea							
Yes	289	170.4 ± 401.0	179.0 ± 560.8	153.7 ± 465.1	8.6 ± 171.4	−25.3 ± 310.1	0.46
No	125	130.9± 1039.6	271.9 ± 1122.5	182.9 ± 750.1	141 ± 537.1	−89 ± 431.5	<0.05
Statin							
Yes	245	201.8 ± 752.9	259.6 ± 933.4	203.5 ± 687.3	57.8 ± 382.9	−56.1 ± 398.3	<0.05
No	169	114.5 ± 352.9	131.0 ± 445.5	103.3 ± 307.2	16.5 ± 142.1	−27.7 ± 271.1	0.54
RAS inhibitor							
Yes	235	238.9 ± 765.7	299.3 ± 941.2	219.1 ± 645.9	60.4 ± 383.0	−80.2 ± 438.8	<0.05
No	179	73.3 ± 289.1	86.0 ± 447.5	88.4 ± 429.2	12.7 ± 140.7	2.4 ± 173.9	0.95
DPP-4i							
Vildagliptin	136	185.6 ± 779.5	245.1 ± 855.5	191.2 ± 618.5	59.5 ± 266.2	−53.8 ± 257.1	0.08
Sitagliptin	96	208.0 ± 181.3	209.0 ± 590.3	143.9 ± 349.2	1.0 ± 88.3	−65.1 ± 479.4	0.66
Linagliptin	77	144.8 ± 776.5	243.2 ± 1073.1	200.4 ± 724.2	98.4 ±54.3	−42.8 ± 45.3	0.30
Saxagliptin	56	233.7 ± 530.9	221.1 ± 688.9	188.8 ± 709.8	−12.6 ± 206.5	−32.3 ± 287.4	0.20
Gemigliptin	48	3.3 ± 27.1	7.2 ± 44.8	12.3 ± 63.6	3.9 ± 28.8	5.1 ± 32.0	0.60

Data are presented as means ± standard deviation.
Some patients were not included in the subgroup analyses due to missing data.
CKD: chronic kidney disease; DPP-4i: dipeptidyl-peptidase IV inhibitor; eGFR: estimated glomerular filtration rate; RAS: renin-angiotensin-system; UACR: urine albumin/creatinine ratio.

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Research Article

Urinary Extracellular Vesicles: Potential Biomarkers of Renal Function in Diabetic Patients

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The aim of this study was to check the relationship between the density of urinary EVs, their size distribution, and the progress of early renal damage in type 2 diabetic patients (DMt2). Patients were enrolled to this study, and glycated hemoglobin (HbA1c) below 7% was a threshold for properly controlled diabetic patients (CD) and poorly controlled diabetic patients (UD). Patients were further divided into two groups: diabetic patients without renal failure (NRF) and with renal failure (RF) according to the Glomerular Filtration Rate. Density and diameter of EVs were determined by Tunable Resistive Pulse Sensing. Additionally, EVs were visualized by means of Transmission and Environmental Scanning Electron Microscopy. Nano-liquid chromatography coupled offline with mass spectrometry (MALDI-TOF-MS/MS) was applied for proteomic analysis. RF had reduced density of EVs compared to NRF. The size distribution study showed that CD had larger EVs (mode) than UD (115 versus 109 nm; $p < 0.05$); nevertheless the mean EVs diameter was smaller in controls than in the CD group (123 versus 134 nm; $p < 0.05$). It was demonstrated that EVs are abundant in urine. Albumin, uromodulin, and number of unique proteins related to cell stress and secretion were detected in the EVs fraction. Density and size of urinary EVs reflect deteriorated renal function and can be considered as potential renal damage biomarkers.

1. Introduction

Recently, the incidence of diabetes mellitus has grown significantly throughout the world and diabetes becomes the most common cause of kidney injury. It is supposed that about 30 percent of patients with diabetes of type 1 (DMt1) and 10 to 40 percent of those with type 2 (DMt2) will suffer from renal damage [1–3]. Most of cells release small membrane spherical structures called extracellular vesicles (EVs) which can be classified into three groups: exosomes (50–100 nm),

microvesicles (100–1000 nm), and apoptotic bodies. These vesicles differ in their composition and subcellular origin. EVs can be found in several body fluids, including plasma, urine, saliva, and milk [4]. In particular, urine is a rich reservoir of these vesicles which originate from the cells facing the urinary lumen (epithelial cells). The urinary EVs can reflect the state of the damage of the kidney. Results of several studies indicate that EVs originating from urine have recently emerged as an interesting source of diagnostic disease biomarkers and contain molecules involved in intercellular communication [5–9]. Changes in excretion rates of specific proteins also can have predictive value in the early diagnosis of renal damage [10].

Existing clinical markers such as serum creatinine or urine albumin level are not very sensitive and are generally increased when acute or chronic renal injury is well established [11]. Reliable biomarkers of renal injury are lacking in the renal care. Creatinine measured by laboratories provides little information about the underlying cause of renal injuries and is less accurate for patients with low muscle mass [12, 13]. In diabetes, the most serious and life treating complication is diabetic nephropathy. To avoid this end stage complication there is a growing need to discover novel noninvasive biomarkers of primary renal damage which allow detecting changes in kidney at early stage [14]. In the present study we test the hypothesis that the density and size of urinary EVs can be considered as biomarkers of renal damage in DMt2 patients.

The motivation of this study was to demonstrate the potential usefulness of urinary EVs in diagnostics of early renal failure as a complication of diabetes. In order to achieve this goal we applied the modern approach for urine analysis: Tunable Resistive Pulse Sensing (TRPS) for EVs enumeration and size distribution analysis, a nano-liquid chromatography technique coupled offline with mass spectrometry (MALDI-TOF-MS/MS) for proteomic analysis and electron microscopy (Transmission Electron Microscopy (TEM); Environmental Scanning Electron Microscopy (ESEM)) for EVs visualization.

2. Materials and Methods

2.1. Study Group. Sixty patients (20 women and 40 men) with type 2 diabetes mellitus (DMt2) were enrolled to the present study. These patients were divided into groups: CD, properly controlled ($n = 24$), and UD, poorly controlled diabetes ($n = 36$). As a control, ten healthy subjects (4 women and 6 men) with an average age of 52 (SD = 7) years were included. The studied groups were allocated according to the criterion of glycated hemoglobin (HbA1c) levels. According to Polish Diabetes Association guidelines from 2014, a HbA1c level of 7% is general criterion of carbohydrate metabolism compensation. Patients in whom HbA1c levels exceed 7% are considered as they have poorly controlled diabetes. What is more, diabetic patients were further classified into two groups: diabetic patients without renal failure (NRF) and with renal failure (RF). A selection of RF was Glomerular Filtration Rate (GFR) below 60 mL/min/1.73 m^2 from MDRD2 formula. Microalbuminuria was defined as 20–200 mg/L and macroalbuminuria >200 mg/L albumin filtration. The clinical characteristics of the studied groups are presented in Tables 1 and 2.

2.2. Urine Samples Collection and Preparation. First morning urine specimens were collected into sterile containers (F.L. Medical SRL, Torreglia, Italy) from diabetic patients and healthy subjects. Typically 50 mL first void urine was used for the isolation of the urinary extracellular vesicles and processed within 2 h of collection. Samples were centrifuged in a Hermle Z300K (Hermle Labortechnik GmbH, Wehingem, Germany) for 10 min in 3000g at 4°C to remove cells and larger debris. After this step supernatants were aliquoted and frozen at −80°C for further analysis. Immediately before the TRPS measurement, samples were thawed in a water bath at 37°C and then vortexed for 30 s, diluted 1 : 1 in PBS (Cat. number P4417, Sigma-Aldrich, St. Louis, USA), vortexed for 10 s, and used for analysis. For mass spectrometry and electron microscopy analysis, supernatants were ultracentrifuged in 150 000g for 1 h at 4°C (Optima™ MAX-XP, Beckman Coulter Life Sciences, Indianapolis, USA) using a horizontal rotor (Cat. number 367280, MLS-50 Swinging-Bucked Rotor, Beckman Coulter Life Sciences, Indianapolis, USA).

2.3. Blood Samples. Blood samples for biochemical and hematology analysis were drawn by venipuncture of the antecubital vein using a 21-gauge needle and the Sarstedt S-Monovette blood collection system (Sarstedt AG & Co., Nümbrecht, Germany) following application of a light tourniquet. For complete blood count analysis and HbA1c levels, EDTA anticoagulant was used. For biochemical analysis, blood was collected in serum separator tubes. Standard blood tests were performed by means of the hematology analyzer (ELITech Group, Puteaux, France). HbA1c level was measured on D-10 analyzer (D-10 hemoglobin testing system, Bio-Rad Laboratories Inc., California, USA).

2.4. Tunable Resistive Pulse Sensing Technology. The size and density of urinary EVs were determined by Tunable Resistive Pulse Sensing (TRPS) technique using qNano system and tunable pore specimen, NP150 from Izon Science (Izon Science Ltd., Christchurch, New Zealand). Principles of the technique were described in [15–18]. To detect particles in the range 60–480 nm the pores labeled NP150 were used. Polystyrene beads of known raw concentration ($1.5E + 13$/mL) and diameter of 105 nm were sourced from Izon Science and were used as a calibrant. Typically a bandwidth filter of 5 kHz was applied during measurements. For the electrolyte and dilution buffer we used PBS. In all measurements 75 μL of electrolyte buffer was placed in the lower fluid cell and the volume in the upper fluid cell was 40 μL. Each sample was measured in triplicate. The density, mean, and mode diameter of EVs are expressed as median (IQR). Data capture was performed using Izon's control suite 3.1 software.

2.5. Proteomics (Nano-LC-MALDI-TOF/TOF Mass Spectrometry). For proteomics analysis urinary EVs were isolated from microalbuminuric (CD) and macroalbuminuric (UD) patients and healthy subjects, at least $n = 3$ from each

Table 1: Clinical characteristics, blood, and urine biochemistry of study groups: C, CD, and UD.

	C $n = 10$	CD $n = 24$	UD $n = 36$	p value
Age (years)	52 (7)	62 (15)†	61 (12)†	0.0683
Gender (male/female)	6/4	17/7	23/13	—
Serum glucose (mmol/L)	5.2 (5.0–5.5)	6.8$^{\dagger *}$ (5.9–7.9)	9$^{\dagger *}$ (7.4–12)	**<0.0001**
Urine albumin (mg/L)	6 (4–13)	6* (2–22)	37$^{\dagger *}$ (12–267)	**<0.0001**
Urine creatinine (mmol/L)	15 (9–17)	5† (4–9)	7† (5–11)	**0.0054**
Serum creatinine (μmol/L)	72 (60–85)	77 (67–98)	79 (62–108)	0.4696
GFR (mL/min/1.73 m^2)	87 (76–101)	77 (59–95)	79 (59–97)	0.5114
EVs density (number/mL)	5.2E10 (2.7E10–1.9E11)	8.4E10 (3.9E10–1.7E11)	5.2E10 (2.6E10–1.5E11)	0.5013
EVs mode diameter (nm)	106 (104–110)	115$^{\dagger *}$ (107–118)	109* (106–112)	**0.0212**
EVs mean diameter (nm)	123 (4)	134 (11)†	129 (8)	**0.0065**

†Significant in comparison with the control group at $p < 0.05$.
*Significant difference between subgroups CD and UD at $p < 0.05$.
Bold means statistically significant difference between the three groups at $p < 0.05$.

Table 2: Clinical characteristics, blood, and urine biochemistry of study groups: C, RF, and NRF.

	C $n = 10$	RF $N = 15$	NRF $N = 45$	p value
Age (years)	52 (7)	69 (11)$^{\dagger *}$	60 (3)$^{\dagger *}$	**0.0027**
Gender (male/female)	6/4	15/3	25/17	—
Serum glucose (mmol/L)	5.2 (5.0–5.5)	8.7† (6.5–11)	7.9† (6.5–10)	**<0.0001**
Urine albumin (mg/L)	6† (4–13)	51† (7–359)	14 (4–58)	0.0923
Urine creatinine (mmol/L)	15 (9–17)	6† (5–8)	8† (4–11)	**0.0046**
Serum creatinine (μmol/L)	72 (60–85)	119$^{\dagger *}$ (111–123)	73* (60–84)	**<0.0001**
GFR (mL/min/1.73 m^2)	87 (76–101)	49$^{\dagger *}$ (39–55)	89* (73–105)	**<0.0001**
EVs density (number/mL)	5.2E10 (2.7E10–1.9E11)	2.6E10* (2.0E10–8.2E10)	8.7E10* (4.0E10–1.9E11)	**0.0361**
EVs mode diameter (nm)	106 (104–110)	111 (105–115)	109 (107–115)	0.1965
EVs mean diameter (nm)	122 (120–126)	129 (123–138)	129† (126–136)	**0.0101**

†Significant in comparison with the control group at $p < 0.05$.
*Significant difference between subgroups RF and NRF at $p < 0.05$.
Bold means statistically significant difference between the three groups at $p < 0.05$.

group. After ultracentrifugation, urine supernatants (6 mL) were used for analysis. Obtained pellet was resuspended in 60 μL 10% SDS (Cat. number L3771, Sigma-Aldrich, St. Louis, USA), 10 μL 1M TRIS (Cat. number T1503 Sigma-Aldrich, St. Louis, USA), and 30 μL deionized water [19]. Protein concentration was determined using BCA method (Cat. number 23227, Pierce Biotechnology, Thermo Scientific, USA). Mean protein concentration was 1.14 ± 1.04 mg/mL; the total protein amount used for MS was 40 μg. Proteomic analysis was performed by means of a nano-liquid chromatograph (EASY-nLC II™, Bruker Daltonics, Germany). The detailed methodology was previously published [20]. The precision tolerance was 100 ppm for peptide masses and 0.7 Da for fragment ion masses. Individual peptide matches with scores above 28 were considered statistically significant. Proteins identification was performed manually, based on two unique peptides with the probability less than 0.05. The protein classification was performed by means of a free algorithm applied in the PANTHER Classification System (Version 11.0, released July 15, 2016) [21]. The analysis of overlapping proteins within healthy subjects, CD, and UD was performed by a tree-circle Venn diagram software [22].

2.6. Transmission Electron Microscopy and Environmental Scanning Electron Microscopy

2.6.1. ESEM. Urine sample from a healthy donor (100 mL) was centrifuged in 3000*g* and next supernatant was ultracentrifuged in 150 000*g* for 1 h at 4°C. EVs pellet was resuspended in 60 μL of PBS and 20 μL of EVs solution was placed on 1 × 1 cm poly-l-lysine slide (Cat. number J2800 AMNZ, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated for 1 h in humid chamber at RT. After incubation the slide was washed twice in PBS and fixed in 3.7% glutaraldehyde in PBS for 30 min followed by salt removal stage. The slide with EVs was washed with two aqueous PBS dilutions, 50% PBS, 25% PBS, and deionized water, each for 1 minute. Next, the dehydration was applied by immersing sample for 30 seconds in ethyl alcohol solutions as follows: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and absolute ethanol. Afterwards, sample was dried for 24 h under cover at RT [23].

The Environmental Scanning Electron Microscopy (ESEM) measurements were performed using SEM Quanta 3D FEG microscope by FEI Company (USA) operated at Institute of Physics Jagiellonian University, Kraków, Poland. The ESEM images were collected by GSED detector using electrons of 5 keV energy. During measurements the specimen was kept at 100 Pa of water vapor at RT.

2.6.2. TEM. Two urine samples from a healthy donor and one UD were prepared in the same way as for ESEM analysis. Samples were centrifuged in Eppendorf tube and fixed with 2.5% glutaraldehyde (Cat. number G5882, Sigma-Aldrich, St. Louis, USA) in 0.1M cacodylic buffer (Cat. number C4945, Aldrich, St. Louis, USA) for 2 h at RT and then postfixed in 1% osmium tetroxide solution (1 hour). Samples were dehydrated by passing through a graded ethanol series and embedded in PolyBed 812 at 68°C.

Ultrathin sections were collected on 300 mesh grids or one slot made from copper. Additionally the latter was covered with formvar film. Next the sections were contrasted using uranyl acetate and lead citrate. For observation the electron microscopy from JEOL company JEOL JEM 2100HT (Jeol Ltd, Tokyo, Japan) was used at accelerating voltage 80 kV.

2.7. CD81 TRIFIc Exosome Assay. Europium Time Resolved Fluorescence assay, Cat. number EX103 (Cell Guidance Systems Ltd., Cambridge, United Kingdom), was used to measure abundance of human CD81 protein in the surface of exosomes in the same urine samples. In the TRIFIc exosome assay the same antibody is used for binding of target to the assay plate and for detection. This assay consists of a monoclonal antibody (labeled with biotin) bound to streptavidin coated plate that captures proteins which are present in the surface of exosomes. An identical monoclonal antibody (labeled with Europium) is used for detection. Europium provides a high degree of sensitivity for the assay. For fluorescence detection we used infinite M200 PRO plate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.8. Statistical Analysis. Statistica 12 (Dell Statistica, Tulsa, USA) and OriginPro 2016 (OriginLab Corporation, Northampton, USA) were used for statistical analyses and plots design. The distribution of continuous data was verified with Shapiro-Wilk normality test. Results are expressed as mean (SD) for data with normal distribution or median and interquartile ranges (Q1–Q3) for data with not normal distribution. Biochemical and epidemiological data were analyzed by one-way analysis of variance ANOVA or Kruskal-Wallis for comparison among groups. Differences between subgroups were tested with Tukey's post hoc test or Dunn's multiple comparison test. The Mann–Whitney *U* test was used to compare differences between two independent groups. Correlations between EVs density and biochemical parameters were calculated with Spearman's rank correlation test, and multiple regression (backward stepwise regression) was performed to predict the effect of age on the other variables. For all analyses p values < 0.05 were considered significant.

2.9. Ethical Considerations. This study was approved by The Bioethical Committee of Jagiellonian University in Kraków on 24 October 2013 which accepted all project's protocols and forms, including an information for patients form and a consent form. The permission number KBET/206/B/2013 is valid until 31 December 2016.

3. Results

A comparison of biochemical parameters such as serum glucose, urine albumin, urine creatinine, serum creatinine, GFR, EVs density, EVs mode, and mean diameter in CD, UD, and the control group was provided in Table 1. Properly controlled diabetic patients (CD) and poorly controlled diabetic patients (UD) had significantly higher levels of serum glucose (6.8 versus 5.2 mmol/L; $p < 0.0001$ and 9 versus 5.2 mmol/L; $p <$

Table 3: Results of Spearman's *rho* test for correlations between EVs density and biochemical parameters.

	C $n = 10$	CD $n = 24$	UD $n = 36$	RF $n = 15$	NRF $n = 45$
Serum glucose	0.49	−0.27	−0.33	**−0.66**	−0.21
(mmol/L)	$p = 0.15$	$p = 0.19$	$p = 0.05$	**p = 0.01**	$p = 0.16$
Urine creatinine	0.08	**0.52**	0.03	−0.46	**0.33**
(mmol/L)	$p = 0.83$	**p = 0.01**	$p = 0.87$	$p = 0.08$	**p = 0.03**
Urine albumin	−0.16	0.37	0.14	−0.03	0.25
(mg/L)	$p = 0.65$	$p = 0.08$	$p = 0.42$	$p = 0.92$	$p = 0.09$
GFR	0.50	0.26	0.27	**−0.54**	0.07
(mL/min/1.73 m^2)	$p = 0.14$	$p = 0.21$	$p = 0.12$	**p = 0.04**	$p = 0.66$

Bold means statistically significant correlation at $p < 0.05$ level.

0.0001) and lower urine creatinine concentration (5 versus 12 mmol/L; $p = 0.003$ and 7 versus 12 mmol/L; $p = 0.004$) in comparison with the control group.

Our results showed statistically significant difference in serum glucose (6.8 versus 9 mmol/L; $p = 0.0001$) and urine albumin (6 versus 37 mg/L; $p = 0.002$) between CD and UD groups. No significant difference was found in serum creatinine concentration, GFR and EVs density between these groups. Size distribution analysis showed that CD had significantly larger EVs mode diameter besides UD (115 versus 109 nm; $p = 0.031$). The mean EVs diameter was smaller in controls than in the CD group (123 versus 134 nm; $p = 0.004$).

A comparison of biochemical parameters in RF, NRF, and the control group is provided in Table 2. Compared with the control group, RF had significantly higher levels of serum glucose (8.7 versus 5.2 mmol/L; $p < 0.0001$) and serum creatinine (119 versus 72 μmol/L; $p < 0.0001$) and lower urine creatinine concentration (6 versus 15 mmol/L; $p = 0.002$) and GFR (49 versus 87 mL/min/1.73 m^2; $p < 0.0001$). NRF had significantly higher levels of serum glucose (7.9 versus 5.2 mmol/L; $p < 0.0001$) and lower urine creatinine concentration (8 versus 15 mmol/L; $p = 0.003$) in comparison with the healthy subjects.

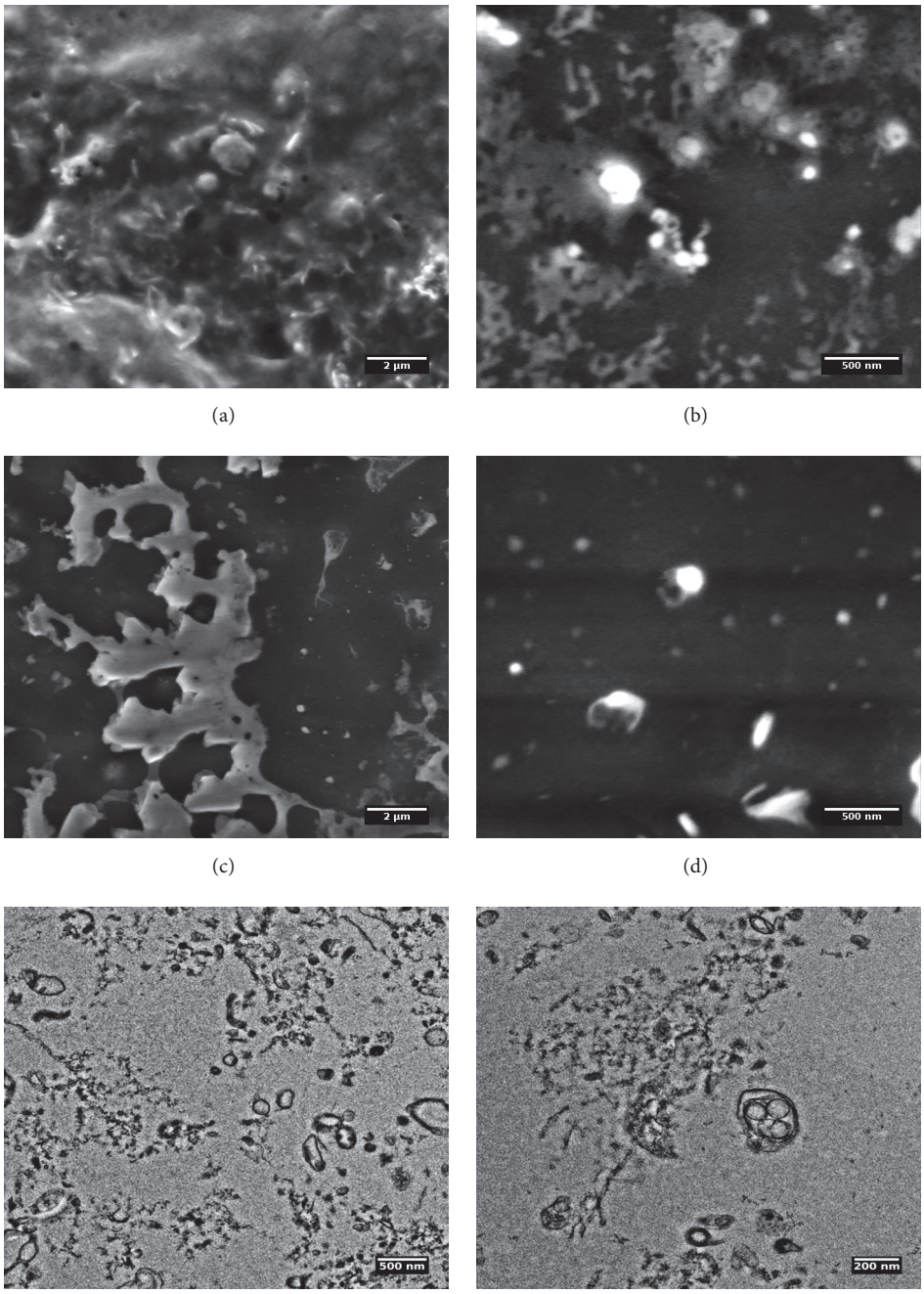
The obtained results indicate that RF had significantly reduced density of EVs compared to NRF (2.57*E*10 versus 8.73*E*10 number/mL; $p = 0.017$). We observed statistically significant difference in serum creatinine (119 versus 73 μmol/L; $p < 0.0001$) and GFR (49 versus 89 mL/min/1.73 m^2; $p < 0.0001$) between RF and NRF groups and in albumin level between RF and healthy subjects (51 versus 6.2 mg/L; $p = 0.02$). Because of high variability within patients groups, no significant difference was found between EVs mode diameters between RF and NRF.

Results of Spearman's *rho* test for relationship between EVs density and biochemical parameters are presented in Table 3. We observed a negative tendency between EVs density and serum glucose level in UD ($R = -0.33$) and negative correlation in RF ($R = -0.66$) patients (Figure 2). There was no correlation between these parameters in CD ($p = 0.19$) and NRF ($p = 0.16$). We found positive relationship between EVs density and urine creatinine concentration in CD ($R = 0.52$) and NRF ($R = 0.33$) (Figure 3). There was no correlation between these parameters in UD ($p = 0.87$) and RF ($p = 0.08$).

Taking into consideration that the age can influence renal function, multiple regression (backward stepwise regression) was performed to show the impact of age on changes in the amount of EVs (see Supplementary Table 1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2016/5741518). Additionally, the correlations of specific biochemical parameters (creatinine, albumin, serum glucose, etc.) with age have been analyzed. Not surprisingly, there was no correlation in control group. The age related negative relationship was observed in CD and NRF group in terms of creatinine clearance (GFR). Such relationship was less significant in patients with more advanced stage of disease (Supplementary Table 2).

Environmental Scanning Electron Microscopy (ESEM) confirmed the presence of the EVs in pellets sedimented after ultracentrifugation of collected samples (Figures 1(a)–1(d)). Washed EVs formed clustered aggregates, which were better distinguishable by means of TEM (Figures 1(e) and 1(f)). The size of EVs was estimated in the range of 130–160 nm. However, a number of smaller and bigger vesicles and other objects were observed.

In order to see the origin and biological activity of analyzed EVs, the proteomic analysis of a urinary EVs fraction was performed. Despite the fact that urine samples were obtained from patients in different stage of DM and different albuminuria levels, the albumin was the main and most abundant protein detected using mass spectrometry methods (Figures 4(a) and 4(b)). The second abundant protein in urine was uromodulin. Venn analysis shows the possible relationships in a protein profile between CD and UD compared to a control subject. Among total 92 proteins in CD, 49 were unique and 31 were common in CD and UD (Supplementary Table 1). In the UD sample, the total number of proteins was 45 while in the control sample 17 proteins were found. The list of unique proteins for every group was listed in Supplementary Table2. For prediction of common protein interactions the list of common 45 proteins was analyzed by means of Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) [24] (Figure 4(b)).



(a) (b) (c) (d) (e) (f)

Figure 1: Environmental Scanning Electron Microscopy (ESEM) (a–d) and Transmission Electron Microscopy (e, f) images of urinary extracellular vesicles (EVs) isolated from a urine sample. ESEM images show that EVs form aggregates and they are clustered on the surface. TEM analysis visualizes the variety of different vesicle-like objects in diameter mostly around 130–160 nm. Interestingly, multivesicle objects were also present in urine that confirms integrity of EVs during preparation.

This analysis revealed the central role of albumin in EVs fraction, nevertheless stress related proteins (ceruloplasmin, transferrin) and cellular components (mostly exosome and extracellular region) (Figures 4(c) and 4(d)).

CD81 TRIFIc exosome assay has not shown any statistical significant differences in CD81 level between study groups, what is presented in Figure 5.

4. Discussion

To date, there are no noninvasive methods to characterize renal structural pathophysiological changes [25]. Moreover, biochemical markers are not sensitive enough to characterize the risk of progression of nephropathy and other DM-related complications [13].

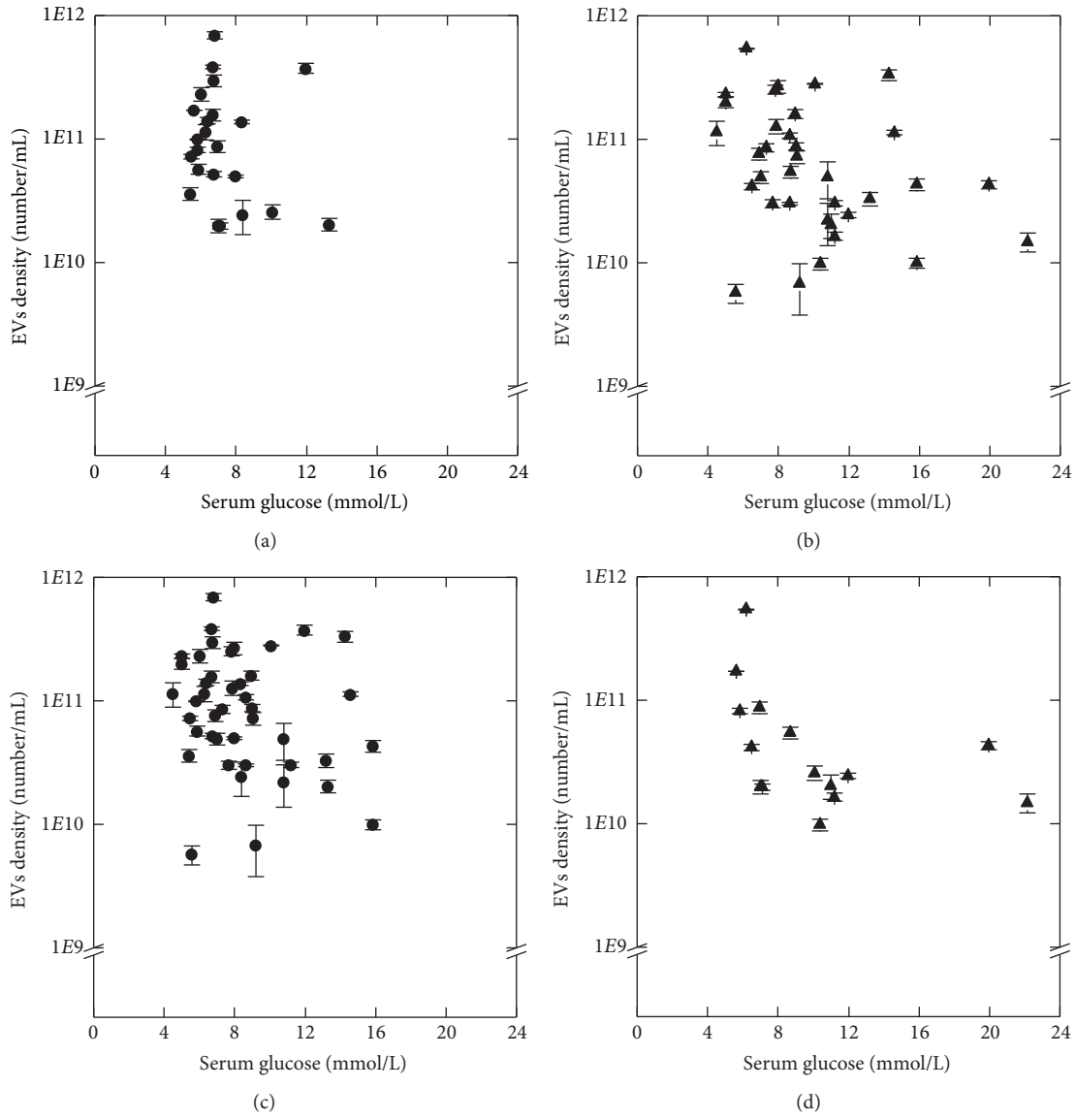


Figure 2: Relationship between EVs density and serum glucose level in study groups: CD (a), UD (b), NRF (c), and RF (d). EVs density values are given as mean (SD). Spearman's rank correlation coefficient, $p < 0.05$.

In this study we sought to test if the density and size of urinary EVs can be considered as potential biomarkers of early renal damage in DMt2 patients which can lead to diabetic nephropathy. Additionally, we studied if there is any correlation between EVs density and biochemical parameters in diabetic patients and healthy control group.

Our results indicate that diabetic patients with renal failure (RF) had lower density of EVs compared to diabetic patients without renal failure (NRF). The size distribution study showed significant difference in EVs mode diameter between CD and UD. Turco et al. [26] showed that decreases of EVs may reflect atherosclerosis and thrombosis-related activity in renal capillaries and parenchyma.

Currently kidney function is monitored by measuring serum creatinine, creatinine clearance, and proteinuria. These clinical markers are usually a late sign of renal damage and indicate its dysfunction [6]. What is more, these markers do not always correlate well with the severity of renal damage seen on biopsy [27]. The early stages of renal functions impairment are diagnosed only by measuring GFR. The complications of chronic renal disease increase with decreasing GFR [28]. There are a number of studies confirming the huge impact of GFR level in progression of renal damage [29–32]. However, a good biomarker of decreased GFR, together with a proper marker of tubular injury, would allow for the diagnosis of renal failure in diabetic patients before increased albuminuria and irreversible kidney damage [13].

One of the specific renal proteins—uromodulin—has been found as urinary biomarker which positively correlates with GFR ratio and decreased uromodulin concentrations have been found in renal failure and diabetic nephropathy [33, 34]. In our study, we observed that uromodulin is a

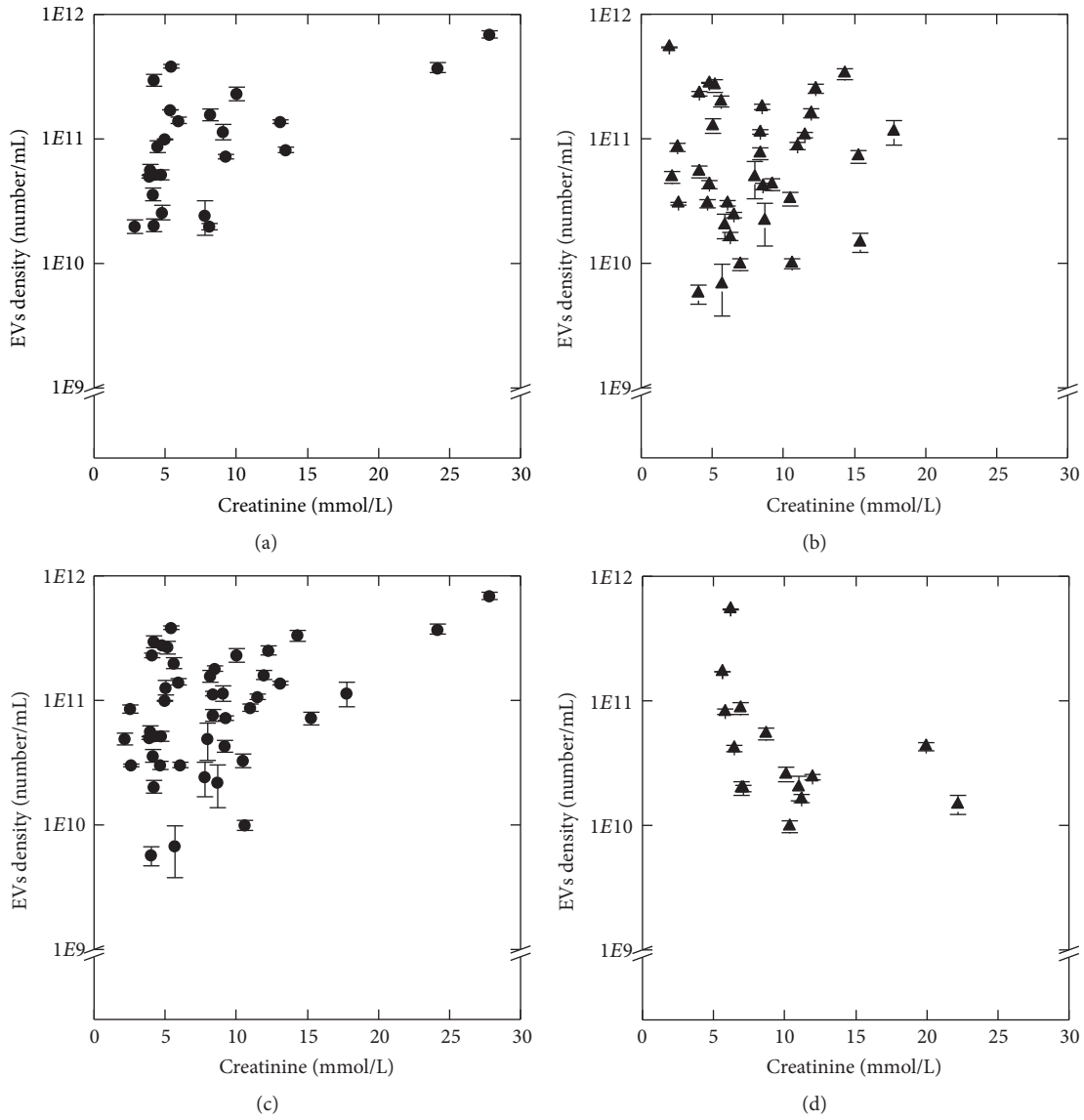


Figure 3: Relationship between EVs density and urine creatinine concentration in study groups: CD (a), UD (b), NRF (c), and RF (d). EVs density values are given as mean (SD). Spearman's rank correlation coefficient, $p < 0.05$.

prominent protein related to EVs and we may assume that its drop in urine concentrations is related to decreased EV density in patient with renal failure. Uromodulin is probably involved in EVs clustering and precipitation (Figure 1(c)) [35].

In our study, we observed a negative correlation between EVs density and serum glucose level in RF and a negative tendency between these parameters in UD. Mehta [36] showed that the kidney is intimately involved in the development of hyperglycemia in the critically ill patients. Sechi et al. [37] demonstrated that abnormal plasma glucose levels were elevated when the GFR was <50 mL/min/1.73 m^2 and overall glucose metabolism parameters were not correlated with microalbuminuria (MA). These data are consistent with our study. We did not observe the correlation between MA and the EVs density in both CD and UD, as well as in RF and NRF. Thus we may assume that EVs presence is more related with impaired glucose metabolism and then with the presence of renal damage biomarker (MA) and EVs can be treated as the more ominous label of disease in diabetic patients. Interestingly, we also observed a positive correlation between EVs density and urine creatinine concentration in NRF and CD, in contrast to those with more advanced disease stage (RF) or impropriate treatment (UD). This observation may suggest that the early renal dysfunction processes are more considerable in the urine EVs release. In the milder stage of renal failure we may expect the higher number of EVs in urine, as the primary marker of the renal dysfunction. In further study the more specific attention should be focused on the correlation between cystatin C and even angiopoietin 2, which appeared to be a relevant predictor of renal dysfunction in acute pancreatitis patients

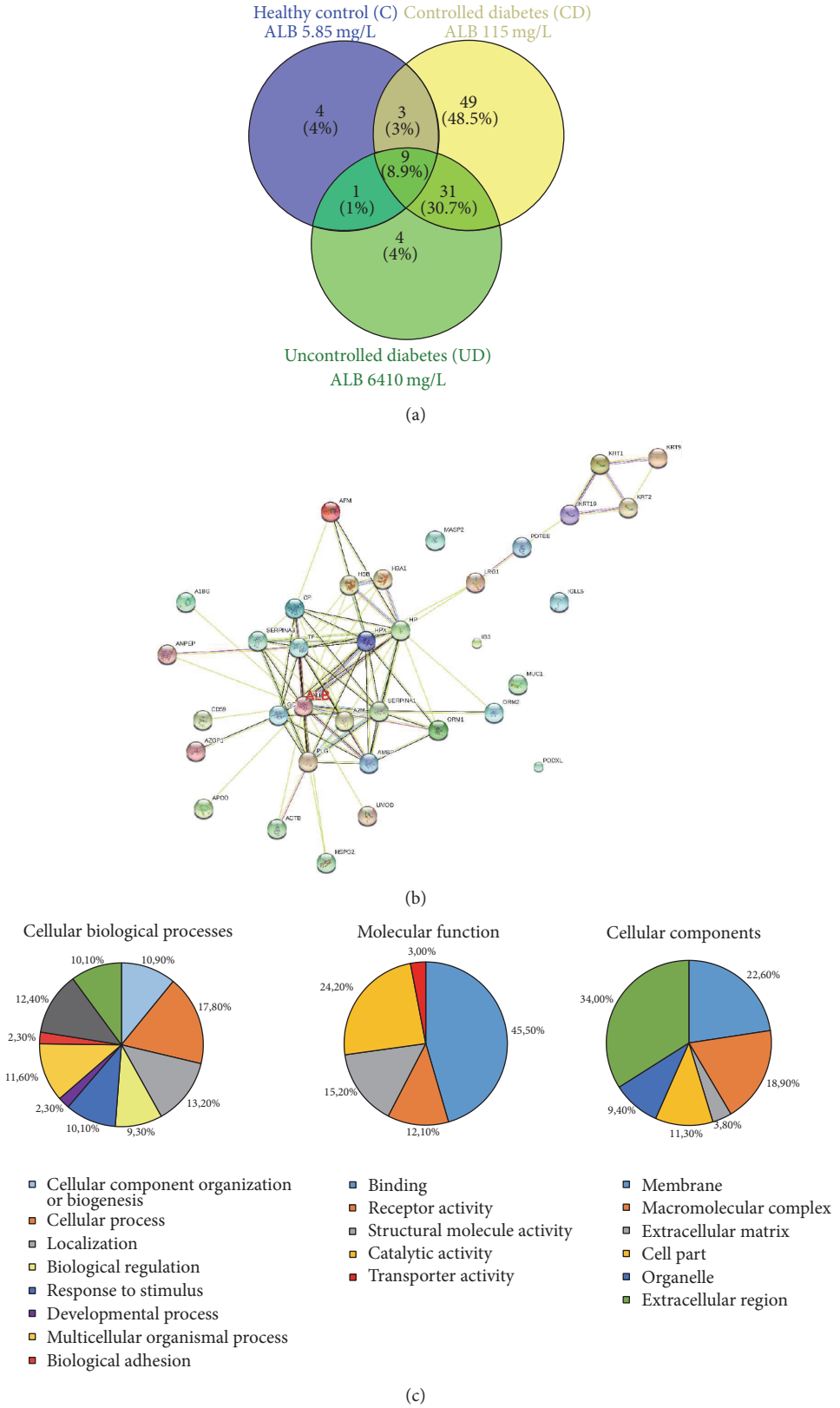
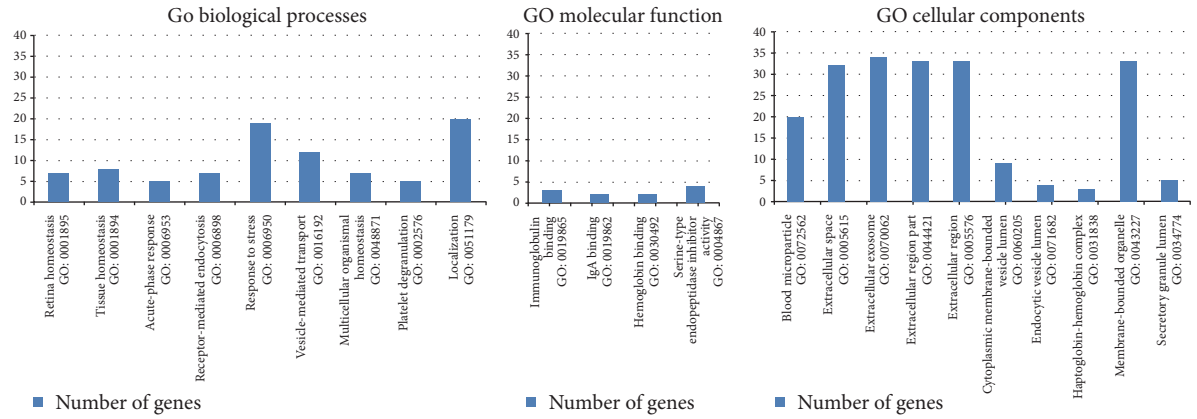


Figure 4: Continued.



(d)

Figure 4: Proteomic analysis of urinary extracellular vesicles: mass spectrometry results from representative samples. (a) Venn diagram shows that in EVs from controlled diabetic patient with microalbuminuria there is a large number of unique proteins ($n = 49$), in a healthy control and in an uncontrolled diabetic patient with macroalbuminuria the number of unique proteins is very low ($n = 4$) [20]. (b) Protein-to-protein interaction analysis of common 45 proteins selected from Venn diagram shows the central role of albumin among urinary EV-related proteins; the list of submitted proteins is available in a supplementary data file (Supplementary Table 2) [22]. (c, d) Gene Ontology analysis showed that most of identified proteins are related to extracellular region or they are related to membrane organelles (exosomes); their localization corresponds with molecular function (receptors and transport proteins) [19]. A1BG: alpha-1-B glycoprotein; A2M: alpha-2-macroglobulin; ACTB: actin, beta; AFM: afamin; ALB: albumin; AMBP: alpha-1-microglobulin/bikunin precursor; ANPEP: alanyl (membrane) aminopeptidase; APOD: apolipoprotein D; AZGP1: alpha-2-glycoprotein 1, zinc-binding; CD59: CD59 molecule, complement; regulatory protein; CP: ceruloplasmin; GC: group-specific component (vitamin D binding protein); HBA1: hemoglobin, alpha 1; HBB: hemoglobin, beta; HP: haptoglobin; HPX: hemopexin; HSPG2: heparan sulfate proteoglycan 2; IGJ: immunoglobulin J polypeptide; IGLL5: immunoglobulin lambda-like polypeptide 5; KRT1: keratin 1; KRT2: keratin 2; KRT9: keratin 9; KRT10: keratin 10; LRG1: leucine-rich alpha-2-glycoprotein 1; MASP2: mannan-binding lectin serine peptidase 2; MUC1: mucin 1; ORM1: orosomucoid 1; ORM2: orosomucoid 2; PLG: plasminogen; PODXL: podocalyxin-like; POTEE: POTE ankyrin domain family member E; SERPINA1: serpin peptidase inhibitor, clade A, member 1; SERPINA3: serpin peptidase; inhibitor, clade A, member 3; TF: transferrin; UMOD: uromodulin.

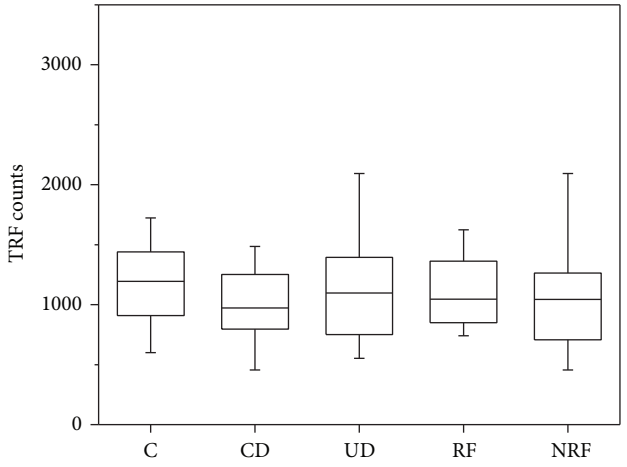


Figure 5: Urine CD81 level. Results from Time Resolved Fluorescence assay. Kruskal-Wallis test: median TRF counts, $p = 0.5$ at $\alpha < 0.05$ significance level.

[38]. Age is the strongest factor influencing physiological state, as well as renal function. According to the number of epidemiological studies, GFR declines with age average about 0.8 mL/min/1.73 m^2 per year. The multiple regression (backward stepwise regression) model did not show the significant impact of age on changes in the amount of EVs.

Urinary EVs are enriched in membrane and cytosolic cargo proteins from the different epithelial cells lining the urinary track. To date, there are only few studies to reveal the new urine biomarkers, which are based on proteomic methods. Among them, proteases and protease inhibitors including kallikreins [6, 10, 14] and metalloproteinases (MMP-2, MMP-7, and MMP-8) have been observed in normoalbuminuria and microalbuminuria groups. In macroalbuminuric patients, cathepsin D has been more abundant than in other patients [14]. In our study we found new protease inhibitors including inter-alpha-trypsin inhibitor (AMBP-Human), inhibitor of the complement membrane attack complex (CD59) and proteases including mannan-binding lectin serine protease 2 (MASP2_Human). A large number of unique proteins in controlled diabetes can be used in selection of potential biomarkers in further studies on the risk of diabetic nephropathy in such patients.

According to our observations the specific marker for exosomes (CD81) did not show significant difference in the level (Figure 5). The CD81 is a surface exosome marker which belongs to the tetraspanin family (TAPA-1) and is involved in signal transduction and cell adhesion [39]. CD81 protein is enriched in the exosome membrane [40]. However, it has not been shown if this biomolecule distinguish the severity of renal disease in diabetic patients. We may also speculate that in our study we did not observe significant differences in CD81 level between groups because the most numerous was

CD81-negative (nonexosomes) population of microvesicles, membrane bubbles above 100 nm (Figures 1(e) and 1(f)).

In our study we confirmed that the quantitative analysis of urinary EVs seems to be a promising tool for defining new biomarkers [41]. We pictured that EVs are present in urine and they maintain their integrity during sampling and preparation process. However, most of these studies are focused on the two areas of progress: bladder or prostatic cancer and acute rejection of renal transplant [10]. Very recent study on diabetic nephropathy novel biomarkers revealed that exosomal regucalcin was underexpressed in renal disease patients [42].

5. Conclusions

Finally we may conclude that urinary EVs have the potential to be biomarkers of renal damage in diabetic patients, and the number of structural and enzymatic proteins (including uromodulin) can be found in urine EVs fraction to be in use as their indicators or new biomarkers both of renal failure and of diabetic nephropathy in the future. The easy accessibility of EVs in urine can increase their use as biomarkers compared to invasive biopsy. Further validation, characterization of the content of bioactive molecules, and larger study population are needed.

Abbreviations

CD: Properly controlled diabetic patients
DMt1: Type 1 diabetes mellitus
DMt2: Type 2 diabetes mellitus
EDTA: Ethylenediaminetetraacetic acid
ESEM: Environmental Scanning Electron Microscopy
EVs: Extracellular vesicles
GFR: Glomerular Filtration Rate
GO: Gene Ontology
HbA1c: Glycated hemoglobin
MA: Microalbuminuria
NRF: Diabetic patients without renal failure
PBS: Phosphate-buffered saline
RF: Diabetic patients with renal failure
TEM: Transmission Electron Microscopy
TRPS: Tunable Resistive Pulse Sensing
UD: Poorly controlled diabetic patients.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Impaired Albumin Uptake and Processing Promote Albuminuria in OVE26 Diabetic Mice

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The importance of proximal tubules dysfunction to diabetic albuminuria is uncertain. OVE26 mice have the most severe albuminuria of all diabetic mouse models but it is not known if impaired tubule uptake and processing are contributing factors. In the current study fluorescent albumin was used to follow the fate of albumin in OVE26 and normal mice. Compared to normal urine, OVE26 urine contained at least 23 times more intact fluorescent albumin but only 3-fold more 70 kD fluorescent dextran. This indicated that a function other than size selective glomerular sieving contributed to OVE26 albuminuria. Imaging of albumin was similar in normal and diabetic tubules for 3 hrs after injection. However 3 days after injection a subset of OVE26 tubules retained strong albumin fluorescence, which was never observed in normal mice. OVE26 tubules with prolonged retention of injected albumin lost the capacity to take up albumin and there was a significant correlation between tubules unable to eliminate fluorescent albumin and total albuminuria. TUNEL staining revealed a 76-fold increase in cell death in OVE26 tubules that retained fluorescent albumin. These results indicate that failure to process and dispose of internalized albumin leads to impaired albumin uptake, increased albuminuria, and tubule cell apoptosis.

1. Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease [1]. Albuminuria is a primary characteristic of DN and a significant predictor for progression towards renal failure [2]. In addition to serving as a marker, albuminuria contributes to the pathology of DN [3]. Controlling the upward progression of albuminuria is a therapeutic goal for preventing decline in renal function [4] of diabetic patients.

Both glomerular protein leakage and impaired tubular protein uptake can contribute to albuminuria. In healthy individuals urine albumin is maintained at low levels by the minimal amount of protein that passes the glomerular filtration barrier and by tubular reabsorption of protein that does pass the glomerular filtration barrier. Extensive literature from humans and animal models supports a role for a defective glomerular filtration barrier in the development of albuminuria [5–7]. There is also evidence that defective protein reabsorption by proximal tubules plays a significant role [8, 9]. However the significance of impaired tubular reuptake and the experimental evidence for this defect is controversial [10]. One recent mouse study demonstrated albuminuria despite the fact that tubular albumin uptake was higher than normal [11].

The diabetic mouse model which manifests the most profound albuminuria is the OVE26 (OVE) transgenic mouse [12]. Their severe albuminuria is valuable for modeling aspects of advanced human DN and for probing the mechanisms of albuminuria. In this report we provide evidence that the high level of albuminuria in OVE mice is due to glomerular leakage combined with reduced uptake in a subset of tubules. Impaired tubular uptake, severe albuminuria, and increased tubule cell death appear to be secondary to their inability to process and eliminate internalized albumin.

2. Methods

2.1. Experimental Animals. OVE diabetic mice on the FVB strain background and FVB controls at 4.5–7 months of age

were bred in our laboratory. All mice had free access to standard chow and water. Procedures were followed per the guidelines of the NIH Guide for the Care and Use of Laboratory Animals and approved by the University of Louisville Institutional Animal Care and Use Committee.

2.2. Urinary Albumin Excretion (UAE). Individual mice were placed in metabolic cages for 24 hours with access to chow and 10% liquid diet (Glucerna, Abbott Laboratories), as previously described [12, 13]. Urinary albumin was determined using a mouse albumin ELISA kit (Bethyl Laboratories).

2.3. Albumin and Dextran Injections and Quantitation. Texas Red and fluorescein-conjugated bovine albumin (TR-albumin and FITC-albumin, resp.) and 70 kDa fluorescein-conjugated dextran (FITC-dextran) were obtained from Invitrogen. The purities of the commercial TR-albumin and FITC-dextran were indicated by the fact that over 98 percent of the fluorescence of the compounds received from the company eluted as a single peak close to bovine serum albumin (BSA) on Sephadex G-75 gel filtration columns (described below). Mice were injected with either TR-albumin, FITC-albumin, or FITC-dextran via the tail vein with a dose of 20 μg/gm body weight. No change in activity was evident in mice for up to four days following albumin or dextran injection. Urine and serum TR-albumin or FITC-dextran were quantified by measuring fluorescence in a Hitachi FU2500 fluorometer at 596/620 nm for TR-albumin or 494/521 nm for FITC. Specimens from noninjected mice of the same type were used to subtract background fluorescence. Sample values were interpolated against known standards of TR-albumin and FITC-dextran. To determine the size of fluorescent fragments in urine, the urine samples were separated into high (>5000 kDa) and low molecular weight components by fractionation on NAP-5 (GE Healthcare) columns. Ten urine samples were separated at higher resolution on an 11.5 cm × 1.5 cm Sephadex G-75 column at a flow rate of 5 mL/hr.

2.4. Histology and Immunohistochemistry. Kidneys were fixed overnight in 4% formalin. Paraffin sections were used for visualization of TR-albumin and FITC-dextran or for immunohistochemistry. For immunofluorescence, sections were blocked with normal serum and incubated overnight at 4°C with goat anti-mouse albumin (1 : 600 dilution, Bethyl Laboratories), rabbit anti-lamp1 (1 : 200 dilution, Abcam), or goat anti-megalin (1 : 50 dilution, Santa Cruz). After three washes in PBS, sections were incubated with secondary antibodies for 1 hour at room temperature, followed by an additional three washes. Donkey secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1 : 50 or 1 : 100 dilution. Images were captured using a Nikon DS-Fi1 camera system with a Nikon E600 microscope.

2.5. TUNEL Staining. Kidney paraffin sections were double stained using mouse albumin antibody and the TUNEL staining kit from Chemicon. Briefly, deparaffinized sections were treated with 10 mM sodium citrate pH 5.0 at 50°C before incubation in Chemicon kit equilibration buffer. This was followed by 60-minute incubation at 37°C with the Chemicon kit terminal deoxynucleotidyl transferase enzyme and reaction buffer. Samples were then incubated with Chemicon kit anti-digoxigenin-FITC conjugate for 1 hr at 22°C. Slides were then stained with goat anti-mouse albumin and AMCA conjugated secondary antibody as described in the preceding paragraph. Sections were observed under a 40x objective by FITC fluorescence for TUNEL stained nuclei in tubule cells. Merged images were made for all FITC positive TUNEL nuclei with albumin staining by AMCA blue fluorescence. From pictures of merged image, counts were made of TUNEL stained nuclei in albumin positive and albumin negative tubule profiles. The number of albumin positive tubule profiles was counted directly over the entire kidney section. The number of albumin negative tubule profiles was estimated by counting the average number of negative tubules in 3 random cortical areas per section and then extrapolating this value to the total cortical area of the section. These values were determined from six different OVE mice and were used to calculate the percentage of albumin positive and albumin negative tubule profiles which contained TUNEL positive tubule cells.

2.6. Statistical Analyses. Data are expressed as means ± SE. Comparisons between two groups were performed by t-test. Correlation was computed as the Pearson product moment correlation coefficient. Statistical analyses were performed with SigmaStat (Systat Software).

3. Results

3.1. Urine Excretion of Injected Fluorescent Albumin or Dextran. Mice were injected with fluorescent Texas Red tagged bovine albumin (TR-albumin). The serum level of TR-albumin was measured in 3 OVE and 3 FVB mice at 10 minutes and 24 hours after injection and found to be similar in the diabetic and normal groups (OVE 14.4 ± 0.6 μg/mL versus FVB 15.9 ± 2.9 μg/mL at 10 minutes and OVE 2.3 ± 0.3 μg/mL versus FVB 2.5 ± 0.8 μg/mL at 24 hours). During the 24-hour period following injection OVE mice excreted more TR-fluorescent tag in their urine than FVB mice (Figure 1(a)). Fractionation (Figure 1(b)) on NAP5 columns revealed that OVE urine contained a 23-fold higher content of high molecular weight TR-albumin, in the first two elution fractions, than was present in FVB urine. Fractionation at higher resolution on Sephadex G75 columns (Figure 1(c)) showed that almost all TR-albumin fluorescence in OVE urine eluted at the position of bovine albumin standard (BSA) and that no TR-albumin eluted near this position in FVB urine. All of the TR-albumin from the manufacturer eluted at the position of BSA (not shown) indicating that small fragments of injected TR-albumin are produced by degradation in mice. Since no full length TR-albumin was detectable by high resolution G75 columns, the 23-fold estimate of increased OVE leakage calculated using NAP5 column data is a minimal estimate. These results also demonstrate that Texas Red tagged bovine albumin behaves similarly to what we reported [12] for endogenous mouse albumin with respect to severe albuminuria in OVE diabetic mice.

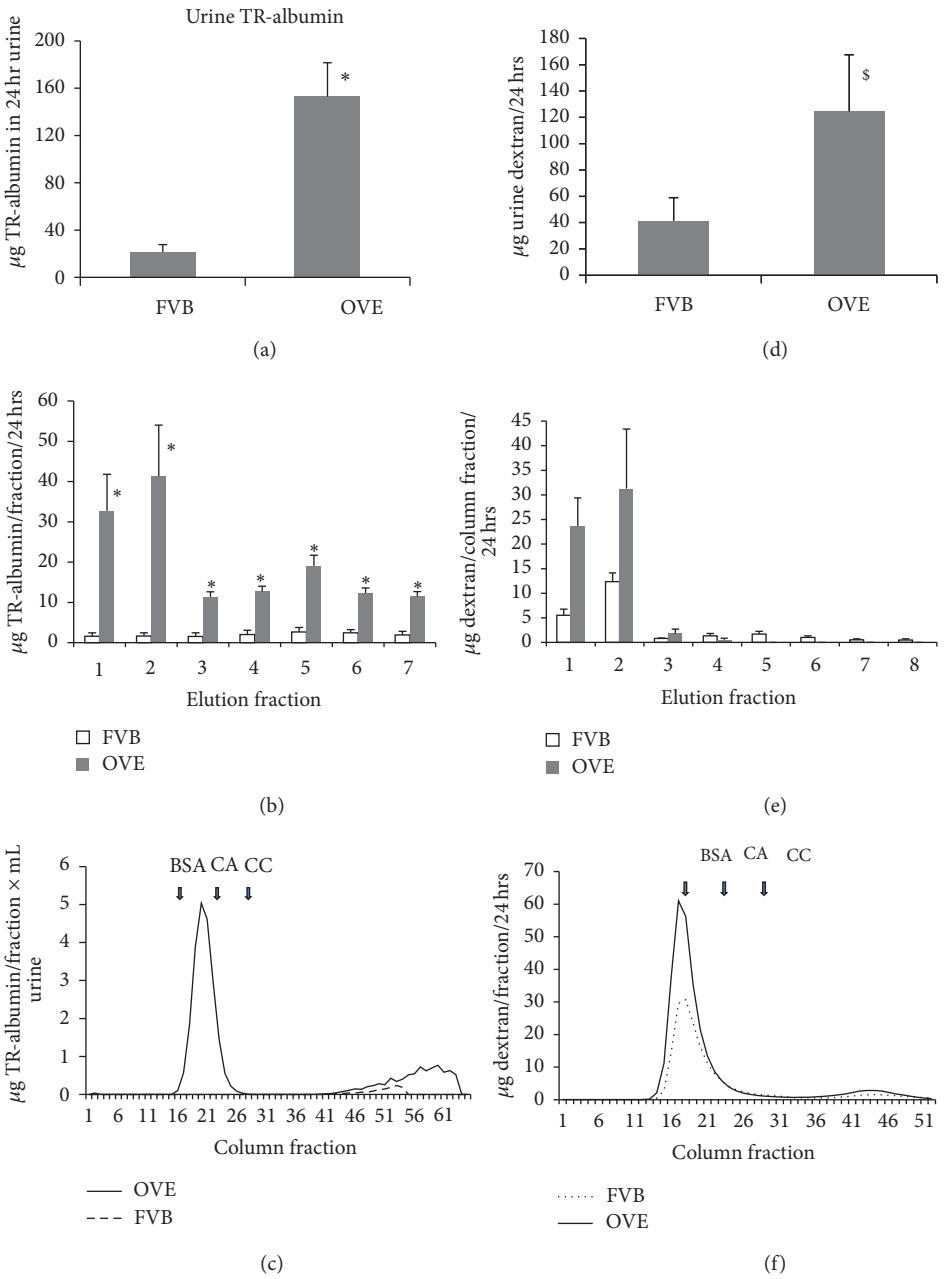

Figure 1: Urine excretion of injected TR-albumin and FITC-dextran. (a) Twenty-four hours after TR-albumin injection, urine samples of OVE mice contain 7-fold more fluorescence than FVB urine. (b) After NAP5 spin column fractionation the first two high molecular weight fractions had 23-fold more fluorescence in OVE urine than in FVB urine. (c) G75 column fractionation profiles of urine show that the high molecular weight peak of OVE urine elutes with the same peak as standard BSA. No TR-albumin peak at this MW could be seen in FVB urine. (d) Twenty-four hours after FITC-dextran injection, urine samples of OVE mice contain 3-fold more fluorescence than FVB urine. (e) After NAP5 spin column fractionation the first two high molecular weight fractions from OVE urine had 3-fold more FITC fluorescence than from FVB urine. (f) G75 column fractionation profiles of urine show that the high molecular weight peak elutes 2 fractions before the standard BSA peak. Arrows indicate peaks for bovine serum albumin (BSA, 66 kDa), carbonic anhydrase (CA, 30 kDa), and cytochrome C (CC, 12 kDa). For panels (a) and (b) results are the average from 4 OVE and 5 FVB mice. Panel (c) results are typical of 3 OVE and 3 FVB mice. Panels (d) and (e) results are the average from 5 OVE and 5 FVB mice. Panel (f) results are typical of 2 OVE and 2 FVB mice. Symbols indicate that OVE values are greater than FVB ($^{*}P \leq 0.02$, $^{\#}P \leq 0.05$, and \$ indicates a trend of $P \leq 0.07$).

Mice were injected with 70 kD FITC tagged dextran to compare the urinary excretion of a compound sieved similarly to albumin, based on size, but subject to different paths of tubular uptake and processing. As shown in Figure 1(d) total 24 hr urine excretion of 70 kDa dextran was 3-fold greater in OVE mice than in FVB mice. Fractionation of the urine on NAP5 columns (Figure 1(e)) or Sephadex G75 columns (Figure 1(f)) showed that almost all dextran in

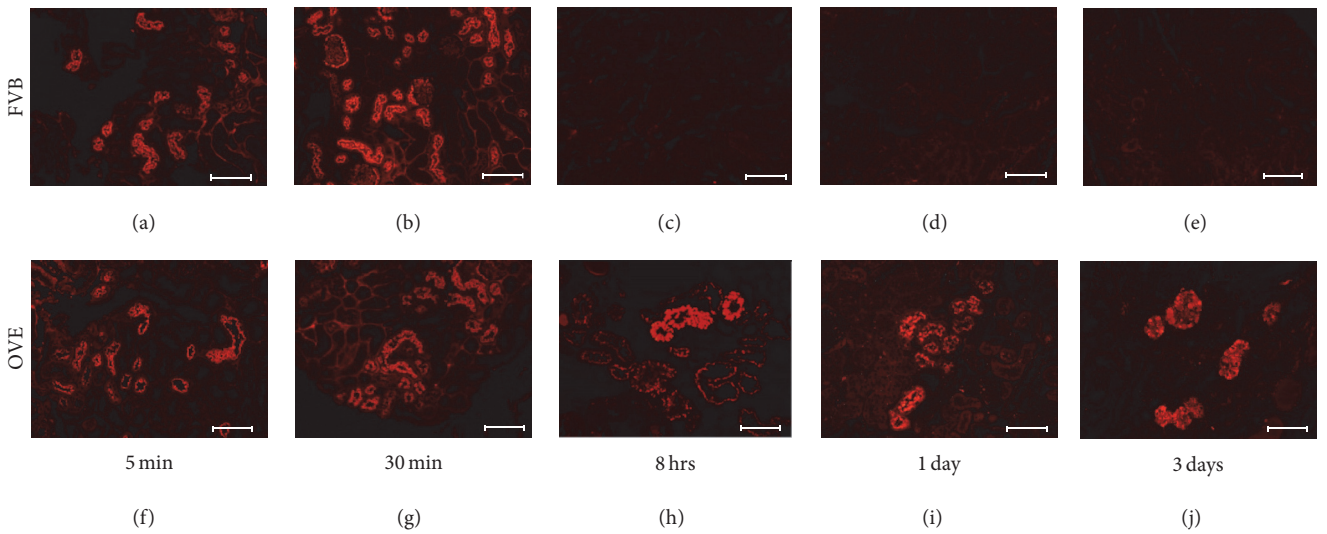

Figure 2: Time course to eliminate TR-albumin fluorescence in FVB and OVE kidneys. Tubule uptake and elimination of injected TR-albumin in FVB and OVE kidneys. Upper panels are from FVB and lower panels from OVE kidneys. Mice were sacrificed at the time points after TR-albumin injection indicated below the images. Initial uptake at 5 min or 30 min is similar in FVB (a, b) and OVE (f, g) tubules. The diabetic and FVB mice differ in that OVE tubules retain TR-albumin for 8 hrs, 1 day, and 3 days (h–j) while no FVB tubules retain TR-albumin at these time points (c–e). Images were made with a 20x objective and the bar in each panel equals 100 μm.

OVE and FVB urine was of high molecular weight. The 3-fold greater excretion of dextran in OVE, calculated from fluorescence in unfractionated urine, was consistent with calculations based on dextran in high molecular weight fractions from NAP5 or Sephadex columns.

3.2. Imaging TR-Albumin in Proximal Tubules. Fluorescence from TR-albumin was used to visualize injected albumin in kidney sections. As shown in Figure 2 TR-albumin that leaked past the glomerulus was concentrated in tubules of OVE and FVB mice within 5 min after injection. Cells that took up TR-albumin were proximal tubule cells as indicated by the presence of megalin in all cells with albumin fluorescence (Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/8749417) and the fact that TR-albumin fluorescence was observed in tubule cells contiguous with Bowman's capsule.

Proximal tubule fluorescence appeared to be similar in OVE and FVB tubules 5 min and 30 min after TR-albumin injection. However, marked differences in tubule fluorescence became obvious at 8 hours and continued 1 day and 3 days after injection. In all FVB tubules the injected albumin was cleared from tubules by 8 hours but in OVE tubules a subset retained strong fluorescence for days after injection. This was despite the fact that fluorescence remaining in serum had dropped to 4% of peak levels after 2 days in OVE and FVB mice. Many of the OVE tubules that retained TR-albumin were dilated and the epithelial cells were enlarged and engorged with albumin (Figures 2(h)–2(j)). Double staining for the endosome and lysosome marker lamp1 demonstrated partial colocalization (Supplemental Figure 2) with FITC tagged albumin 24 hours after injection. This pattern has been reported for lamp1 and endogenous albumin in normal mice [14].

3.3. Loss of Albumin Uptake in Tubules That Could Not Clear Albumin. We previously reported that large amounts of mouse albumin accumulate in a subset of OVE proximal tubulesz [15]. To determine how accumulation of albumin in OVE proximal tubule cells affected subsequent protein reabsorption we compared uptake of TR-albumin to uptake of endogenous mouse albumin (Figure 3). Most OVE tubules that stained for accumulated endogenous mouse albumin (Figure 3(a)) did not take up injected TR-albumin (Figures 3(b) and 3(c)). Examination of 445 tubule cross-sections that stained strongly for mouse albumin from 4 OVE mice revealed that only 16% of these cross-sections showed uptake of injected TR-albumin. This suggests that prior accumulation of albumin impairs the ability of proximal tubule cells to take up TR-albumin.

To estimate the time scale for loss of albumin uptake OVE mice were injected with fluorescent albumin 2 days apart: first with TR-albumin, followed 2 days later by a second injection of FITC tagged albumin (Figure 4). In these mice almost none of the tubules that retained fluorescence from the first TR-albumin injection (Figure 4(a)) stained for FITC-albumin from the second injection (Figures 4(b) and 4(c)). Therefore, tubules that had taken up and retained TR-albumin for 2 days were then unable to take up FITC-albumin. Since tubules with prolonged retention of TR-albumin were defective in their ability to take up additional albumin we examined whether TR-albumin retention was associated with the severity of albuminuria. As shown in Figure 5 the number of tubule segments that retained injected TR-albumin fluorescence for 2-3 days was significantly correlated with albuminuria.

3.4. Cell Death in Proximal Tubule Cells That Retain Albumin. TUNEL staining was performed to determine if albumin accumulation increased the rate of tubule epithelial cell death

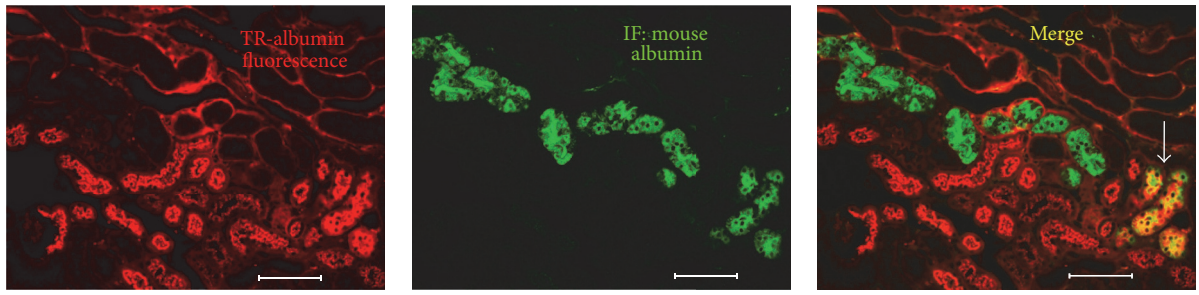

Figure 3: TR-albumin uptake and endogenous mouse albumin accumulation in OVE tubules. Most OVE tubules that take up TR-albumin, injected 5 min before sacrifice (image (a)), are not the same OVE tubules that accumulate endogenous mouse albumin, shown in image (b) by immunofluorescence (IF) staining for mouse albumin. In the merged image (c) only 3 tubule profiles are yellow indicating costaining for mouse albumin and TR-albumin (shown by the white arrow). Images were made with a 20x objective and the bar in each panel equals 100 μm.

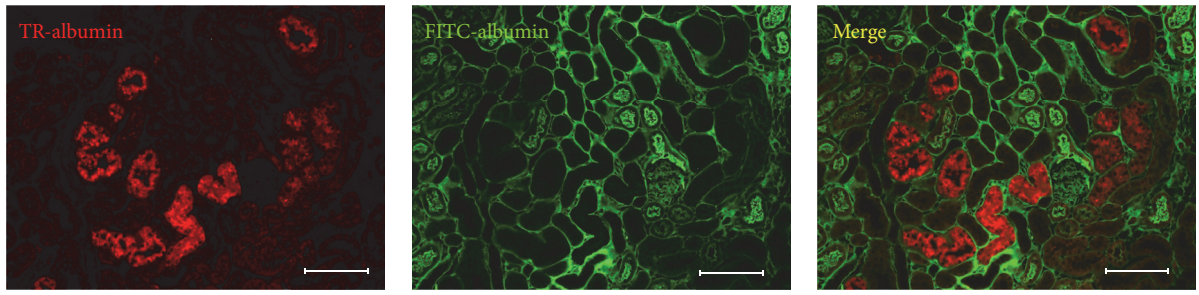

Figure 4: OVE tubule fluorescence for albumins injected 2 days apart. OVE tubules that retain TR-albumin fluorescence for two days after injection (panel (a)) do not take up FITC-albumin (panel (b)) injected, 5 min before sacrifice. In the merged image (panel (c)) no tubules are double stained. Images were made with a 20x objective and the bar in each panel equals 100 μm.

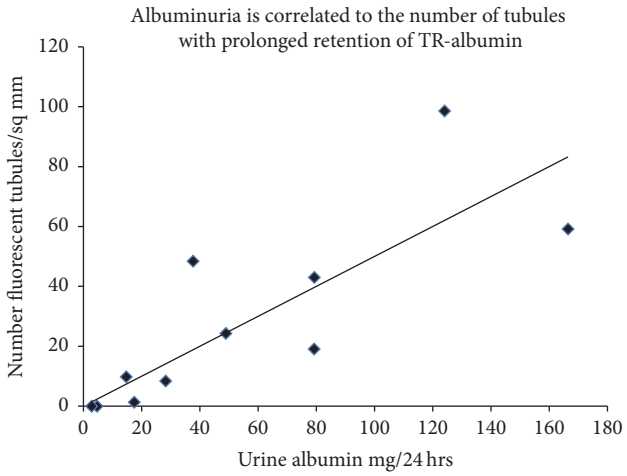

Figure 5: There is a significant correlation between albuminuria and the number of tubules with prolonged retention of TR-albumin. Each data point is from one OVE mouse and shows 24 hr urine albumin excretion of that mouse and the number of tubule segments counted with TR-albumin fluorescence per sq cm of kidney cortex 48 to 72 hours after the mouse was injected with TR-albumin. Pearson product correlation coefficient is 0.81, $P \leq 0.01$ ($n = 11$). The trend line shows the best fit linear regression (slope = 0.48).

(Figure 6). TUNEL staining in epithelial cells was rare in OVE tubules and even less frequent in FVB tubules (data not shown). In OVE tubules that accumulated albumin, the frequency of TUNEL staining was 76-fold greater than in OVE tubules with no albumin accumulation.

4. Discussion

The diabetic model with the most severe albuminuria is the OVE diabetic model [16] but the basis for the severity of proteinuria is unknown. There is a long standing question [10, 17] about the significance of impaired proximal tubule protein reuptake in diabetic albuminuria. Most studies have focused on defects in diabetic glomeruli, especially podocytes [13, 18]. Podocyte effacement, mutations in podocyte genes, or loss of podocytes results in increased glomerular permeability and leakage of albumin [19]. However Russo et al. [17] and Comper and Russo [20] have proposed that impaired tubular reabsorption of albumin is the primary cause of diabetic albuminuria. Our results in diabetic OVE mice show that both glomerular leakage and impaired tubular reabsorption contribute to the severity of OVE diabetic proteinuria.

Albumin and 70 kDa dextran have similar glomerular sieving coefficients [17] resulting in similar passage through

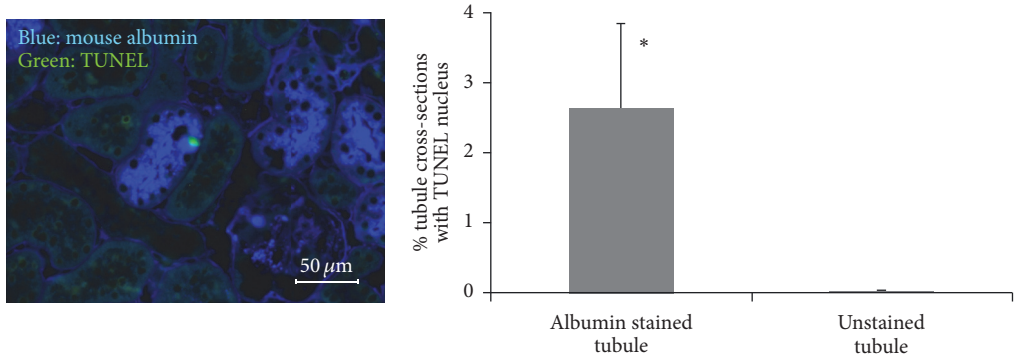

Figure 6: TUNEL stained cells are increased in OVE tubules that accumulate albumin. (a) Merged image of mouse albumin immunostaining (blue) and TUNEL staining (green) on an OVE section. (b) Quantitation of TUNEL staining in tubules with and without albumin staining from the same OVE kidney sections. Only TUNEL staining in tubular epithelial cells was counted. TUNEL stained cells within the tubule lumen or between tubules were not counted. Complete sections from 6 OVE mice were counted. $*$ indicates $P < 0.05$. TUNEL staining was performed as described in Section 2. The image was taken with a 40x objective and the bar indicates 50 μm.

the glomerular filtration barrier. Albumin is taken up by proximal tubule cells by specific mechanisms [14, 17] not used by dextran. If OVE proteinuria is due only to increased glomerular pore size, then leakage of dextran and albumin should be similarly affected. Conversely, if proteinuria is mostly due to impaired tubule uptake then leakage should be greater for albumin and leakage should be less for dextran. Results in Figure 1 demonstrate that diabetic urine contained at least 20-fold more injected bovine albumin than normal urine but only 3-fold more 70 kDa dextran. The greater impact of OVE diabetes on urine albumin compared to dextran suggested a more significant role for impaired tubule uptake. However, since output of both albumin and dextran was increased, then both increased glomerular leakage and impaired tubule uptake were implicated in OVE proteinuria.

Fluorescent tagged albumin provided a visual marker for tubule cell uptake and disposal of albumin. From 5 to 30 minutes after albumin injection there was no apparent difference between images of OVE and FVB mouse proximal tubule fluorescence (Figure 2). However after 8 hours the difference was obvious. By this time all FVB tubules and most OVE tubules were free of fluorescence. However a few OVE tubules retained strong TR-albumin fluorescence and continued to fluoresce for as long as 3 days after injection. Evidently, these long-fluorescing tubule cells could take up the injected TR-albumin but were unable to process and eliminate it. One day following injection of FITC tagged albumin, merged images of injected albumin and the protein lamp1, a marker for endosomes and lysosomes, demonstrated partial colocalization in OVE tubules (Supplemental Figure 2). The pattern was similar to what has been reported in normal tubules [14] and suggests that albumin was moving along normal processing pathways despite slow or interrupted processing. Proximal tubule cells apparently have a higher capacity to take up albumin than they can process. When OVE tubule cell processing capacity is exceeded they are unable to adequately suppress excessive uptake and accumulation of albumin continues. The most likely cause of too much proximal tubule cell uptake is very high concentrations of albumin in the tubule lumen from leaking glomeruli. We previously described structural abnormalities in the OVE glomerular filtration barrier [12, 13, 21–25] and functional results showing that a subset of OVE glomeruli have severe leakage of albumin [15]. Supplemental Figure 3 shows that OVE glomeruli still filter TR-albumin, despite possessing obviously abnormal podocyte organization, as revealed by a podocyte GFP transgene [26].

OVE mice were given consecutive injections of fluorescent albumin, two days apart. Tubules that retained fluorescence two days after the first albumin injection showed no fluorescence from the second albumin injection (Figure 4). This result indicates that tubules unable to fully process and eliminate internalized protein rapidly lose the ability to continue to take up albumin. We also found that albumin uptake was impaired in OVE tubules that were engorged with endogenous mouse albumin (Figure 3). Therefore experiments with injected fluorescent albumin and endogenous mouse albumin both showed that failure to clear internalized albumin interferes with subsequent albumin uptake. However, our methods were unable to determine if the tubules were permanently impaired. Conceivably some tubules may regain function. Permanently disabled tubules would lead to a progressive decline in the number of functional nephrons in the kidney, ultimately leading to renal failure. OVE tubules unable to take up protein provide an unobstructed pathway for intact albumin to pass into the urine. The significance of these abnormal tubules to total albuminuria is supported by the strong correlation (Figure 5) found between tubules unable to clear fluorescent albumin and the level of albuminuria in OVE mice.

The fact that some tubule cells were engorged with albumin, unable to clear internalized albumin, and impaired in albumin uptake indicated that these cells were not healthy and potentially predisposed to apoptosis. This was tested by TUNEL staining to identify dying cells. Within the same OVE kidney, tubule cell death was 76-fold higher if the tubule cells

contained accumulated albumin than if they did not contain albumin (Figure 6). In a model of profound, nonselective proteinuria, Motoyoshi et al. [27] also found excessive apoptosis in tubule cells that accumulated cytoplasmic protein droplets. They attributed the increase in apoptosis to uptake of very high molecular protein, though their results could have been equally explained by intracellular protein accumulation. Tubular accumulation of albumin is found in biopsy specimens from human diabetics, which we have confirmed [15] in multiple diabetic biopsy specimens. OVE mice and advanced DN patients share similar pathologies of tubule albumin accumulation and urine excretion of large amounts of intact albumin [9].

5. Conclusions

Elevated urine output of injected dextran and greater output of injected albumin indicated that the severe albuminuria found in OVE diabetic mice was due to a combination of glomerular leakage and failure of tubules to reabsorb albumin. Visualization of fluorescent tagged albumin revealed a subset of OVE tubules unable to dispose of internalized albumin even after several days, which led to protein engorgement. This was associated with and probably a cause of impaired albumin uptake, increased albuminuria, and increased tubule cell death. Insufficient ability to process and dispose of internalized protein by proximal tubule cells promotes the progression of diabetic nephropathy.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Y. S. Long and S. Zheng contributed equally to this work.

Acknowledgments

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Research Article

Reversal of Early Diabetic Nephropathy by Islet Transplantation under the Kidney Capsule in a Rat Model

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Objective. Diabetic nephropathy (DN) is a common microvascular complication of diabetes mellitus, and insulin therapy has many side effects in the treatment of DN. Islet transplantation has emerged as a promising therapy for diabetic patients. This study was established to investigate its advantageous effects in a rat model of early DN. *Methods*. Streptozotocin was administered to the rats to induce diabetes. Twelve weeks later, the diabetic rats were divided into 3 groups: the islet-transplanted group (IT group), the insulin-treated group (IN group), and the untreated group (DN group). Renal injury and kidney structure were assessed by urinalysis and transmission electron microscopy (TEM) detection. Immunohistochemical staining and western blotting were performed to assess renal fibrosis levels. *Results*. The early DN features were reversed and the glomerular filtration barrier and basement membrane structures were improved at 4 weeks after islet transplantation. The urine microalbumin-to-creatinine ratio (ACR), protein-to-creatinine ratio, and mean thickness of the glomerular basement membrane (GBM) were significantly decreased in the IT group. The expression of renal fibrotic factors was also significantly decreased. *Conclusions*. These data suggest that early DN can be reversed after islet transplantation, and they may facilitate the development of a clinical therapeutic strategy for human diabetes mellitus.

1. Introduction

Diabetic nephropathy (DN) is one of the common microvascular complications of diabetes, and it is characterized by expansion of the mesangial matrix, thickening of the glomerular basement membrane (GBM), persistent proteinuria, and progressive renal dysfunction in diabetic patients with hyperglycemia [1]; it has been estimated that approximately 20%–30% of type 1 and type 2 diabetic patients develop DN [2]. It is considered the leading cause of end-stage renal disease (ESRD) in many developed countries, and, in China, it accounts for more than 25% of all cases of ESRD [3]. The occurrence and development of DN seriously affect the quality of life, prognosis, and even survival of diabetic patients [4, 5].

Components of the high glucose milieu in diabetes are the main causes of renal injury in DN; therefore, controlling the plasma glucose level is considered the most crucial goal during treatment. In addition to patients with type 1 diabetes mellitus, those with type 2 diabetes might also eventually require insulin therapy [6]. An increasing number of studies have found that although diabetic patients start taking medication or insulin at the early disease stage, most of them still develop progressive DN, indicating that appropriate therapeutic targets and additional treatments are urgently needed [7]. In the past few decades, with a general understanding of diabetes mellitus, various therapeutic protocols have been proposed to treat diabetes mellitus or its late complications, including vascularized, whole-pancreas transplantation and islet cell transplantation [8]. Recent studies have suggested

that pancreas or islet transplantation can ameliorate the clinical signs of diabetes mellitus, stabilize further progression, and even reverse the related renal injury in early DN [9–11], primarily because it is more suitable for use under physiological conditions *in vivo*. Nevertheless, the complexity of the vascularized whole-pancreas transplantation procedure may, along with the preexisting diseases present in many diabetic patients, increase the risks of operative and postoperative complications [7].

Recently, with gradual advances in methods of islet isolation, islet cell transplantation has progressed from the laboratory to the clinical setting [12]. Compared with whole-pancreas transplantation, it is a simpler operation with lower morbidity, and it has become an attractive alternative therapeutic method to conventional insulin injection or pancreas transplantation for treating diabetes and its complications [13]. The purpose of this study was to investigate the efficacy of islet transplantation for reversing early DN and to establish whether this treatment is superior to insulin therapy for the regression of DN. The differences between islet transplantation and pancreas transplantation are also discussed in our study.

Furthermore, commonly measured parameters were assessed, as described in previous studies, including the blood glucose level, body weight, and the urine protein level. Microscopic pathological changes of the glomerulus and podocytes during treatment were also examined. Podocyte abnormalities are crucial components of progressive renal injury and are known as good predictors of early DN [12]. Studies of the role of podocytes in renal injury have highlighted their importance in recent years. In this study, we have elaborated upon the significance of the recovery of podocyte structure and function during the treatment of diabetic nephropathy. Additionally, the roles of profibrogenic factors and interstitial cytokines, including TGF-β1, HGF, CTGF, α-SMA, and MCP-1, during the progression of renal fibrosis were assessed in early DN. The links between these fibrotic factors and the possible mechanisms of podocyte injury in early DN are also discussed in this study.

2. Materials and Methods

2.1. Animal Model and Groups. A total of 50 male Sprague-Dawley (SD) rats weighing 200–220 g were provided by the Experimental Animal Center of Wenzhou Medical University, and some of the rats ($n = 12$) were used as islet donors. All rats were housed with a 12 h light/dark cycle at 24°C ± 1°C and provided with food *ad libitum* for a week before initiation of the study. All animal experiments were approved by the Wenzhou Medical University management Committee for Medical Laboratory Animal Sciences. A rat diabetic model was induced by a single peritoneal injection of streptozotocin (STZ) (55 mg/kg of body weight) in sodium citrate buffer (pH 4.5) after fasting overnight. The plasma glucose concentration was measured in a drop of tail vein blood using an Accu-Chek glucometer (Roche Diagnostics, Indianapolis, IN). One week later, when the nonfasting blood glucose concentration was ≥16.67 mmol/L for 3 days, the experimental diabetic rat model was established [14]. After 12 weeks, the urine ACR and protein-to-creatinine ratio were determined, and transmission electron microscopy (TEM) was performed to assess whether the early DN rat model had been successfully established. Next, the rats were randomly divided into three different groups: in the untreated group (DN group, $n = 8$), the rats were left untreated and studied 4 weeks later; in the islet-transplanted group (IT group, $n = 8$), the rats underwent islet transplantation under the kidney capsule and were studied 4 weeks later; and, in the insulin treatment group (IN group, $n = 8$), the rats were given glargine insulin (Wanbang Pharmaceuticals, Jiangsu, China) by subcutaneous injection at 9 a.m. and 9 p.m. every day for the following 4 weeks (3 U each). The normal control rats were regarded as the control group (NC group, $n = 8$).

2.2. Islet Isolation and Purification. Islets were isolated from the rat pancreas using previously described methods [15]. Briefly, the rats were anesthetized by intraperitoneal injection of chloral hydrate, and laparotomy was performed to expose the pancreas. The entrance of the common bile duct into the intestine was located and ligated, and 8 mL collagenase V (0.8 mg/mL, dissolved with Hanks solution) was injected into the common bile duct by retrograde intubation. When the pancreas was fully inflated, it was separated from the surrounding tissues with surgical tweezers, transferred to a 50 mL centrifuge tube, and digested for 10–15 min at 37 ± 0.5°C. After digestion, the tissue was washed with Hanks' solution three times. Then, the islets were purified by density gradient centrifugation (Histopaque -1119 and Histopaque -1077) at 2000 rpm for 5 min. The supernatant was poured into a new centrifuge tube and transferred to a black glass culture dish for manual selection of islets. The final purified islets were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Invitrogen, Inc., USA) at 37°C and 5% CO_2.

2.3. Islet Counting, Equivalent Calculation, and Activity Evaluation. The purified islets were adjusted to an appropriate concentration in the culture medium and transferred to a small culture dish with a 2 mm lattice for their quantification under a microscope. Based on the methods of Lembert [16] and others, the cell clusters were counted, and the diameters were measured with a microscope eyepiece scale. Total islet equivalents (IEQ) were calculated according to the appropriate formula [16]. A single aliquot of 100 freshly isolated islets was aspirated into a 200 μL pipette tip and transferred to a small culture dish. The activity of the islets was evaluated by fluorescein diacetate-propidium iodide (FDA-PI) staining under an inverted fluorescence microscope. The activity ratio was determined using 100 islet cell clusters to estimate that of the total final purified islets.

2.4. Islet Transplantation under the Kidney Capsule. Based on the method reported by Napoli et al. [17], the final purified islets of approximately 800–1000 IEQ were aspirated into a 1 mL syringe connected to P50 polyethylene tubing, and the islet was transferred to the head end. The recipient rat was anesthetized by intraperitoneal injection of chloral hydrate, the left flank was shaved, and the kidney was exposed through

a small lumbar incision. Capsulotomy of the kidney was performed on the caudal outer surface, and the tip of the polyethylene tubing was inserted and advanced gently under the kidney capsule. The surface of the kidney was kept moist with saline during the procedure. The islet in the tubing was pushed out slowly and carefully, and the tube was removed after complete transfer of the islet into the capsule.

2.5. Tissue and Urine Sampling. The nonfasting blood glucose levels and body weights of the rats in each group were measured once per week. Individual rat metabolic cages were used to collect random and 24 h urine samples twice per week. The urine protein concentration was measured using the sulfosalicylic acid precipitation method, and the creatinine concentration was determined with a creatinine assay kit (Bioassay Systems, Hayward, CA). Albuminuria was determined using a rat albumin-specific ELISA kit (Exocell Laboratories, Philadelphia, PA). The small pieces of renal cortex obtained from the kidney tissue were cut into small cubes and fixed in 2.5% glutaraldehyde. The remaining tissue was fixed overnight in 4% paraformaldehyde for histopathological staining. After gradient alcohol dehydration and incubation with xylene transparent agent, the kidney tissue was embedded in paraffin and cut into 5 μm sections using a microtome.

2.6. Immunohistochemistry Staining. Following the IHC staining protocol described by Pichaiwong et al. [18], the paraffin tissue sections were incubated overnight with primary antibodies to TGF-β1, HGF, CTGF, α-SMA, MCP-1, and synaptopodin at 4°C. Then, they were incubated with secondary antibodies and visualized with diaminobenzidine (DAB; brown color) and hematoxylin counterstaining under a microscope. The intensity of positive staining was determined according to the IOD/area value with Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Silver Spring, MD). More than 10 fields for each section in each group were evaluated, and the mean value was used. All scoring was performed on blinded slides.

2.7. Immunofluorescence Staining. Immunofluorescence staining was performed using a rabbit anti-insulin polyclonal antibody to measure the activity of transplanted islets under the kidney capsule. The secondary antibody used for this analysis was changed to goat anti-rabbit IgG-FITC, and the nucleus was stained with Hoechst staining solution.

2.8. Transmission Electron Microscopy (TEM). Small pieces of the renal cortex tissue block were fixed in 2.5% glutaraldehyde and washed with PBS (0.01 M). Then, they were postfixed with 1% osmium tetroxide, dehydrated with an acetone gradient, and embedded in Araldite M (Sigma Aldrich). Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined with a transmission electron microscope (H-7700, Hitachi, Japan). The thickness of the GBM was measured with Image-Pro Plus 6.0 image analysis software.

2.9. Western Blotting Analysis. The proteins were extracted, and the total protein concentration was determined by Protein BCA Assay (Beyotime, Jiangsu, China). After blocking with 5% skim milk, the membranes were incubated overnight at 4°C with the primary antibodies. Then, they were incubated with goat anti-rabbit IgG (1 : 5000) conjugated to horseradish peroxidase (HRP) and visualized with an ECL Western Blotting Detection System (Amersham, Arlington Heights, IL, USA).

2.10. Statistical Analysis. All statistical data were analyzed using SPSS version 19.0 statistical software (SPSS, Chicago, IL USA), and the values are expressed as the mean ± standard deviation. Multiple comparisons between groups were performed by one-way ANOVA, and *post hoc* analyses were conducted using the least significant difference test. The differences between groups were considered significant at a $p < 0.05$.

3. Results

3.1. Evaluation of the Early DN Rat Model. As shown in Figure 1, the urine microalbumin-to-creatinine ratio (ACR) and protein-to-creatinine ratio were determined to assess renal injury, and transmission electron microscopy (TEM) detection of the kidney tissue was performed to identify pathological changes. As local GBM thickening, podocyte depletion with fusion of foot processes, mesangial expansion, disordered endothelial cell arrangement, and glomerular filtration barrier structure abnormalities were evident, and the mean ACR (2.73 ± 0.58 mg/mmol) and urine protein-to-creatinine ratio (66.14 ± 7.25 mg/mmol) were significantly higher compared to those in the NC group (0.28 ± 0.06 mg/mmol and 14.37 ± 1.17 mg/mmol, resp. (Figures 1(c) and 1(d)), $p < 0.05$); the early DN rat model was considered to have been established successfully, as described previously [19]. Thus, this rat model was considered suitable for evaluating the advantageous effects of islet transplantation on early DN.

3.2. Islet Transplantation and Evaluation. Before transplantation, islet activity was evaluated by FDA-PI staining with an aliquot of islets, and the results revealed a high level of islet activity (>99%, Figure 2(b)). The transplanted islets were distributed and were visible under the capsule (Figure 2(a)). At four weeks after transplantation, immunohistochemical and immunofluorescence staining for insulin demonstrated high activity of the transplanted islets under the kidney capsule and indicated that they were still capable of normal insulin secretion (Figures 2(c) and 2(d)). Throughout this experiment, the surviving islets retained normal insulin secretion function and maintained the blood glucose level within the normal range; immunofluorescence staining verified these results.

3.3. Blood Glucose, Body Weight, and Urinary Parameters. At three days after transplantation, the blood glucose level in the IT group was significantly decreased and was maintained at a normal level (5.96 ± 1.81 mmol/L, Figure 1(a)). The body weights of the rats in the IT and IN groups gradually

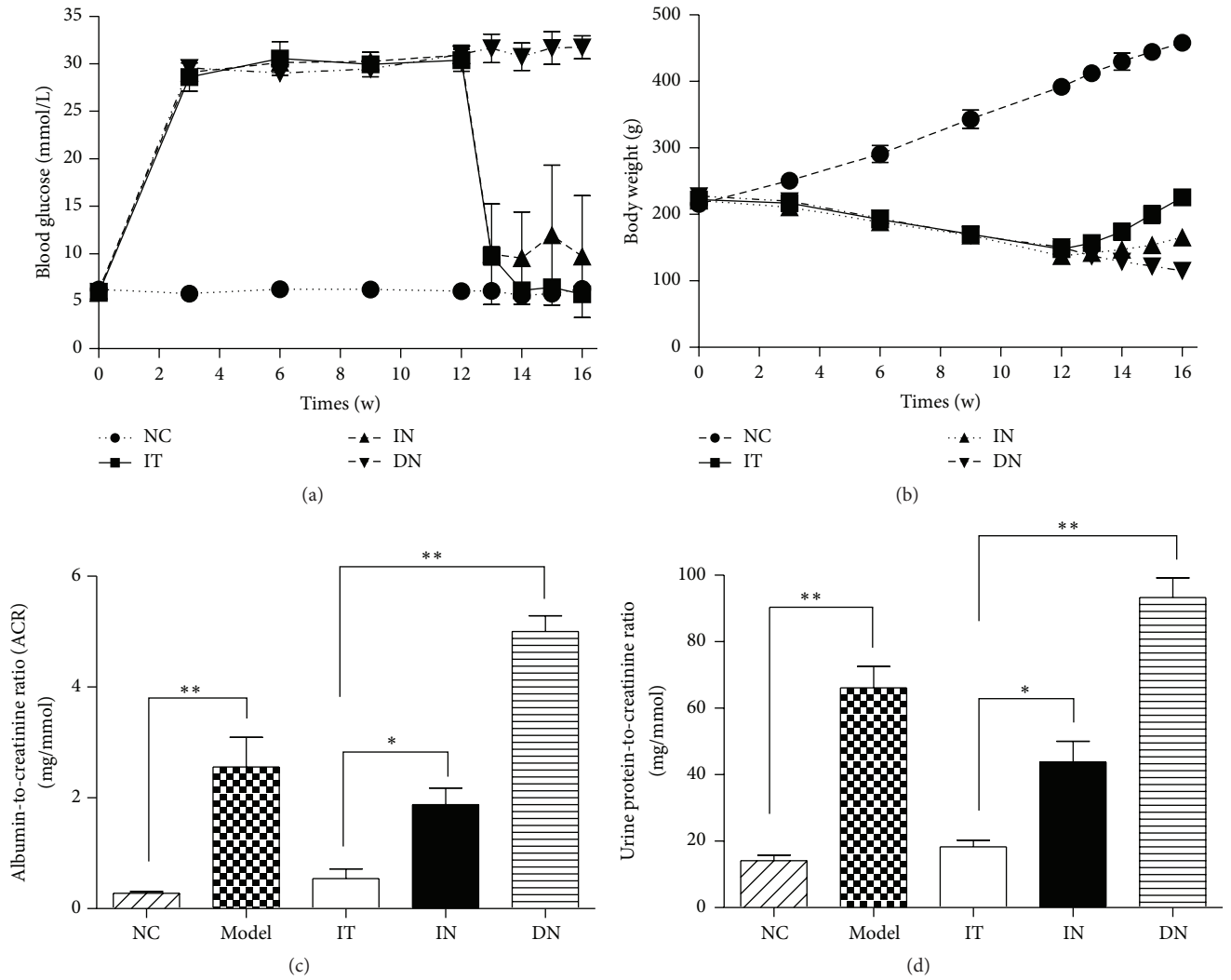

Figure 1: Blood glucose levels and body weights over 16 weeks and urinalysis results for each group. (a) Nonfasting blood glucose levels for each group. (b) Body weight changes over 16 weeks. (c) Random urine albumin-to-creatinine ratios (ACR) for each group, $^{*}p = 0.002$ and $^{**}p < 0.001$. (d) Urine protein-to-creatinine ratios for each group, $^{*}p = 0.001$ and $^{**}p < 0.001$. Model group: diabetic nephropathy model rats established at 12 weeks.

increased, whereas the growth curve for the IT group was significantly steeper than that for the IN group (Figure 1(b)). The glucose level in the rats in the DN group continued to remain high, along with a continuous decrease in body weight. As shown in Figure 1(c), after 4 weeks, the urinary ACR decreased significantly in the IT group (0.43 ± 0.11 mg/mmol) after islet transplantation. In contrast, it remained high in the IN group (1.64 ± 0.49 mg/mmol). Compared to the model group (2.73 ± 0.58 mg/mmol), the urinary ACR was higher (5.18±1.36 mg/mmol) in the DN group. The urine protein-to-creatinine ratio also obviously differed between the IT and IN groups (Figure 1(d)). Compared with the IN (43.76±6.30 mg/mmol) and DN groups (93.43 ± 8.51 mg/mmol), there was a significant decline in this ratio, which dropped close to the normal level (14.37 ± 1.17 mg/mmol), in the IT group (17.95 ± 2.20 mg/mmol).

3.4. TEM Examination of Glomerulus and Podocyte Density Evaluation. As shown in Figure 3(a), we found that the thickening of the GBM, disordered endothelial cell arrangement, and podocyte depletion with fusion of the foot processes were not obvious in the IT group compared with the NC group. In contrast, in the IN and DN groups, the range of podocyte depletion with fusion of the foot processes was more extensive than that in the IT group (Figure 3(a)). Additionally, as shown in Figure 3(c), the mean thickness of the GBM was measured, and the data demonstrated that the thickness in the IT group (0.24 ± 0.02 μm) was significantly less than that in the IN group (0.33 ± 0.05 μm, $p = 0.001$) or DN group (0.46 ± 0.04 μm, $p < 0.001$). Synaptopodin stains podocytes specifically, and podocyte density can be measured by the intensity of synaptopodin staining. As shown in Figure 3(b), the intensity of synaptopodin staining was significantly

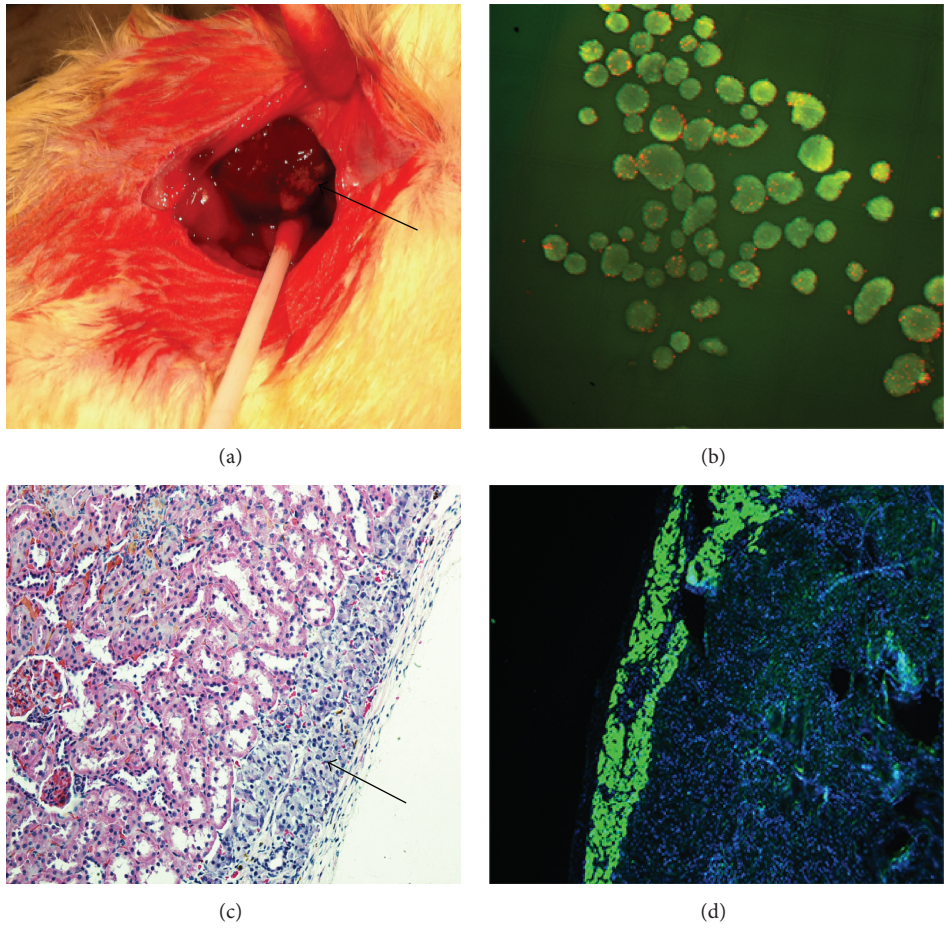

Figure 2: Islet transplantation under the kidney capsule and evaluation of islet activity. (a) Islets transplanted into the renal subcapsular space. (b) Activity evaluation of isolated islets (FDA-PI staining, ×100). (c) Transplanted islets under the kidney capsule (HE staining, ×200). (d) Insulin immunofluorescence staining (green, ×100).

decreased in the model and DN groups compared with the NC group. The strength of synaptopodin staining was obviously greater in the IT group than in the IN group, indicating a greater density of podocytes. This was also confirmed by the greater IOD/area value of synaptopodin staining in the IT group compared with the IN group ($p = 0.003$), as shown in Figure 3(d).

3.5. Assessment of Renal Fibrosis. To confirm and compare the ameliorative effects of islet transplantation and insulin therapy on renal fibrosis in early DN, immunohistochemical staining was performed to determine the expression of fibrosis-related factors. As shown in Figures 4 and 5, decreased expression of TGF-β1, CTGF, MCP-1, and α-SMA and increased expression of HGF were detected in the IT group compared with the IN group ($p < 0.05$ for each). Additionally, the brown staining of the granules was significantly decreased in the IT group compared with the DN group, and the IT group also had a lower mean IOD/area value for the related renal fibrosis factors ($p < 0.001$). Western blotting analysis further demonstrated that the protein expression of each factor was obviously lower in the IT group compared with the IN and DN groups (Figure 6); these results coincide with the immunohistochemical staining results.

4. Discussion

Over the past few decades, islet transplantation has emerged as an alternative therapeutic method for the use of conventional antidiabetic drugs and insulin for the treatment of diabetic patients [20]. Several studies have demonstrated that pancreas or islet transplantation is an effective treatment for diabetic patients with complications. In recent years, remarkable results have also been observed in the clinical application of islet transplantation in humans. Researchers have demonstrated that the kidney graft survival rates for uremic patients with type 1 diabetes mellitus can improve significantly after successful islet transplantation [21]. These findings further demonstrated that islet transplantation is an effective treatment for diabetic nephropathy. Our study was designed to investigate the efficacy of islet transplantation for reversing early DN and to establish whether this treatment is superior to insulin therapy for treating diabetic complications in model rats. The early DN model was successfully

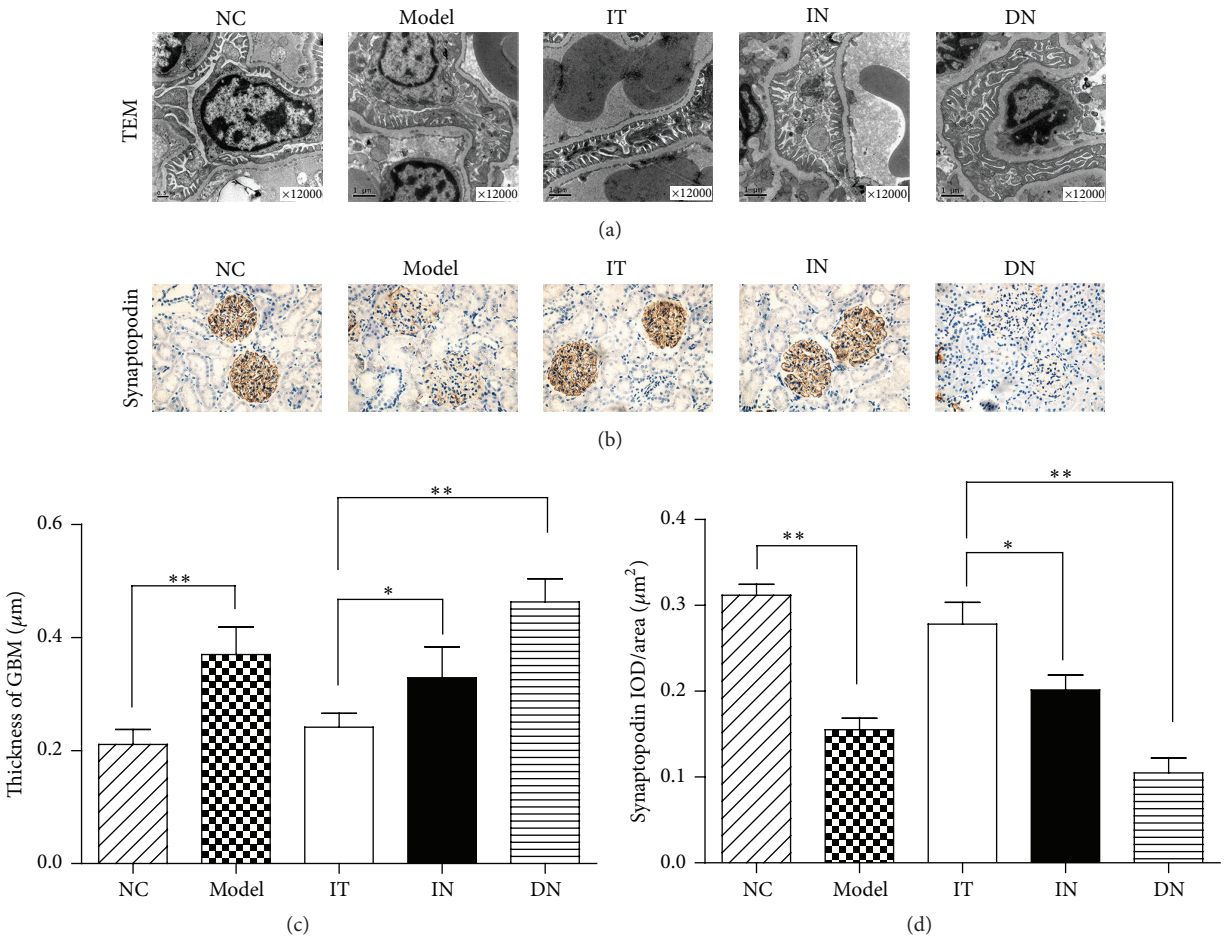

Figure 3: TEM detection of renal tissues and immunohistochemical staining for synaptopodin in podocytes. (a) TEM detection of podocytes and the glomerular filtration barrier. (b) Immunohistochemical staining for synaptopodin, which stains podocytes specifically, to measure the density of podocytes. (c) Measurement of the thickness of the GBM in each group, $^{*}p = 0.012$ and $^{**}p < 0.001$. (d) Comparison of the IOD/area of synaptopodin immunohistochemical staining for each group, $^{*}p = 0.003$ and $^{**}p < 0.001$. IOD/area, integrated optical density/area. The mean of IOD/area of positive staining was used to accurately measure the degree of positive expression in immunohistochemical staining. Model group: diabetic nephropathy model rats established at 12 weeks.

established as described previously [19]. Throughout the study, the transplanted islets were able to maintain the blood glucose level within the normal range without the use of immunosuppressants in SD rats with diabetic nephropathy. These findings are in agreement with those of Ar'Rajab and colleagues [13]. The results of our study demonstrated that all of the measured clinical signs of diabetes mellitus were abolished after islet transplantation and that renal injury was significantly improved; these signs included a decrease in the blood glucose level, attenuation of proteinuria and albuminuria, and normalization of the glomerular filtration barrier. Further, histopathological staining revealed that renal fibrosis was obviously alleviated after islet transplantation. In contrast, with insulin treatment, the blood glucose level, ACR, and protein-to-creatinine ratio were not well controlled, and the expression of related renal fibrosis factors was obviously higher than that observed following islet transplantation in this study. All of these data demonstrated that early DN could be reversed after islet transplantation, and the ameliorative effects achieved with transplantation were significantly better than those obtained with insulin therapy for the treatment of diabetic complications. Furthermore, TEM was also used in this study to detect microscopic changes of the glomerular basement membrane (GBM) and podocytes in the glomerulus. Wide application of this technology would allow for more accurate analysis of kidney diseases in the clinical setting. Changes of microscopic structures (podocytes and GBM) are considered to play an important role during the progression of renal disease.

Podocytes are highly differentiated epithelial cells in the glomerulus that attach to the GBM to maintain efficient glomerular filtration. Their interdigitating foot processes are bridged by a slit diaphragm to control the patency through the transcytotic clearance mechanisms [22]. Recent research has indicated that podocyte damage plays a crucial role in progressive DN and that it can directly result in progressive renal hypofunction [22, 23]. In the early stage of diabetes, podocyte dysfunction is manifested histologically by the

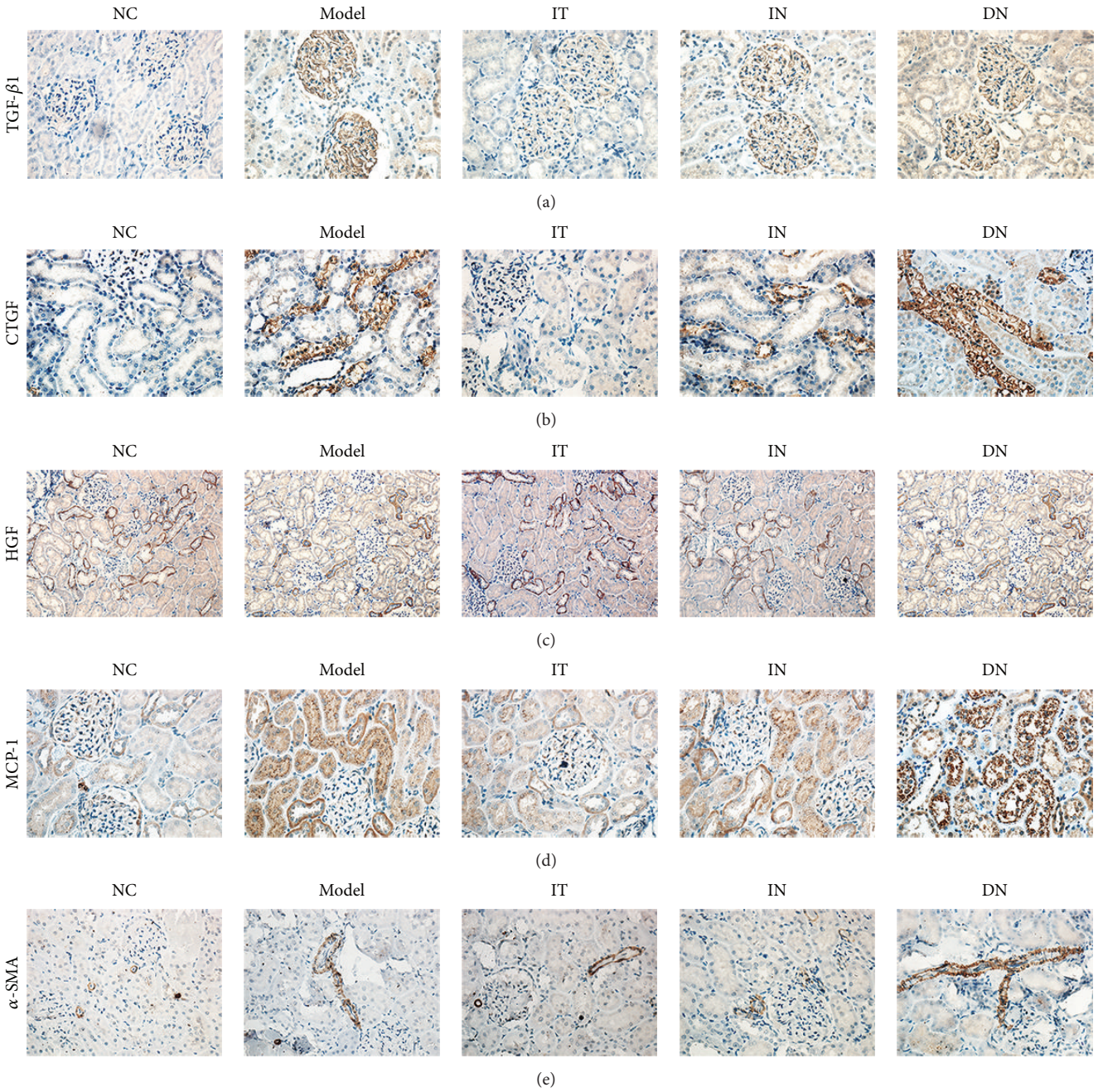

Figure 4: Immunohistochemical staining of renal fibrogenic and interstitial fibrosis factors in each group. (a) TGF-β1. (b) CTGF. (c) HGF. (d) MCP-1. (e) α-SMA. Model group: diabetic nephropathy model rats established at 12 weeks.

broadening of foot processes and functionally by various physiological alterations. Thickening of the GBM is also an important characteristic of the progression of DN. Injury to the GBM or podocytes could cause barrier damage and protein loss, which might explain why progressive proteinuria was the typical manifestation of early DN [24]. In our study, islet transplantation significantly ameliorated the thickening of the GBM and the podocyte depletion with fusion of the foot processes. These results coincided with the improvement in renal function and attenuation of kidney damage. Growing evidence indicates that the signaling abnormalities in podocytes in DN mainly involve the TGF-β family [25]. The blockage of inflammation-induced injury or intervention of inflammatory mechanisms in podocytes could ameliorate renal fibrosis in early DN [26].

Renal fibrosis is one of the typical features of early DN, and the degree of fibrosis is strongly associated with the progression of DN [27]. Progressive accumulation of myofibroblasts in the glomerulus and tubules is critical for the development of DN; therefore, early prevention or

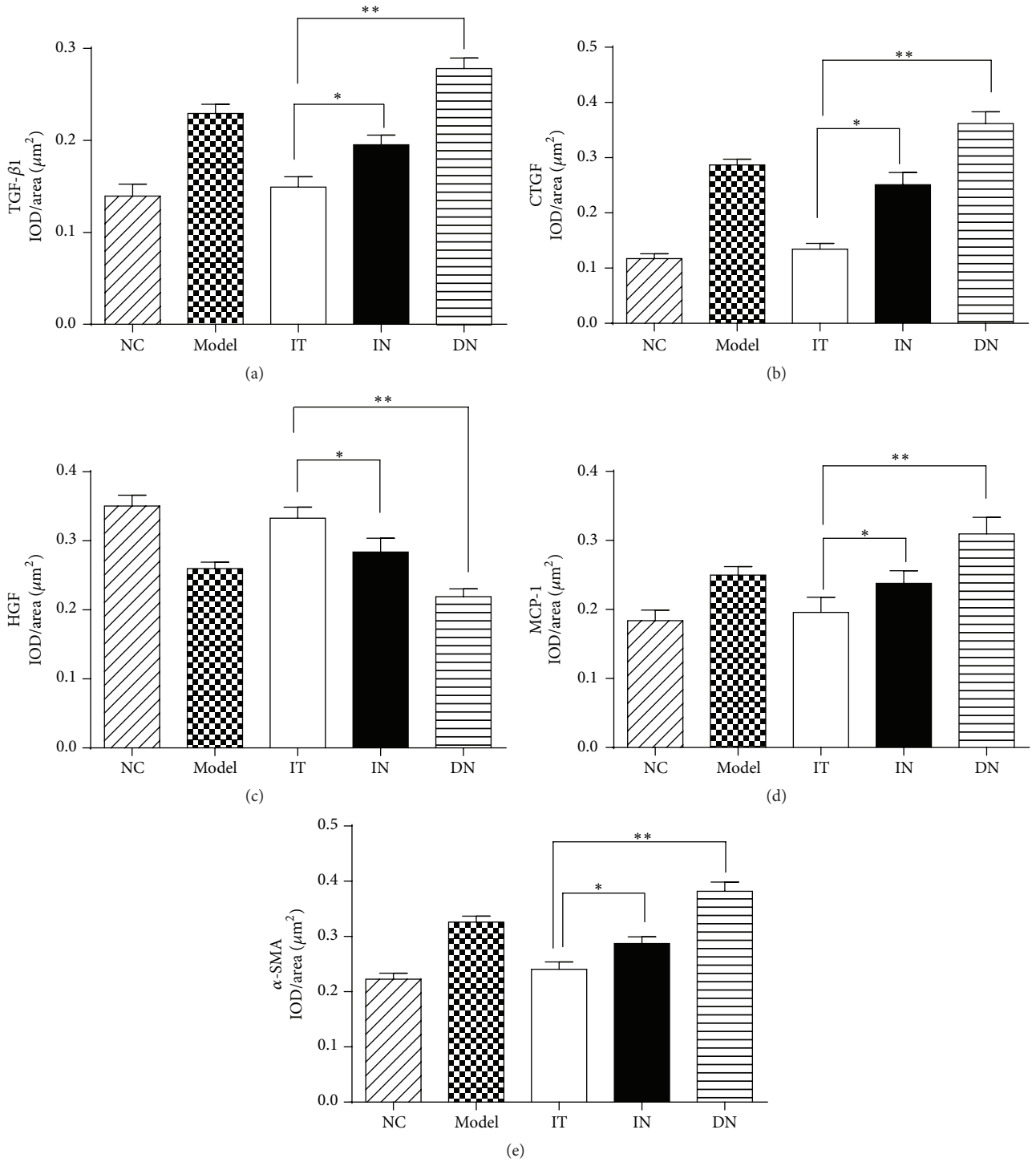

Figure 5: Measurement of IOD/area of immunohistochemical staining of renal fibrogenic and interstitial fibrosis factors. (a) Measurement of IOD/area of TGF-β1, $^{*}p = 0.013$, and $^{**}p < 0.001$. (b) Measurement of IOD/area of CTGF, $^{*}p = 0.002$ and $^{**}p < 0.001$. (c) Measurement of IOD/area of HGF, $^{*}p = 0.001$ and $^{**}p < 0.001$. (d) Measurement of IOD/area of MCP-1, $^{*}p = 0.001$ and $^{**}p < 0.001$. (e) Measurement of IOD/area of α-SMA, $^{*}p = 0.018$ and $^{**}p < 0.001$. IOD/area, integrated optical density/area. The mean of IOD/area of positive staining was used to accurately measure the degree of positive expression in immunohistochemical staining. Model group: diabetic nephropathy model rats established at 12 weeks.

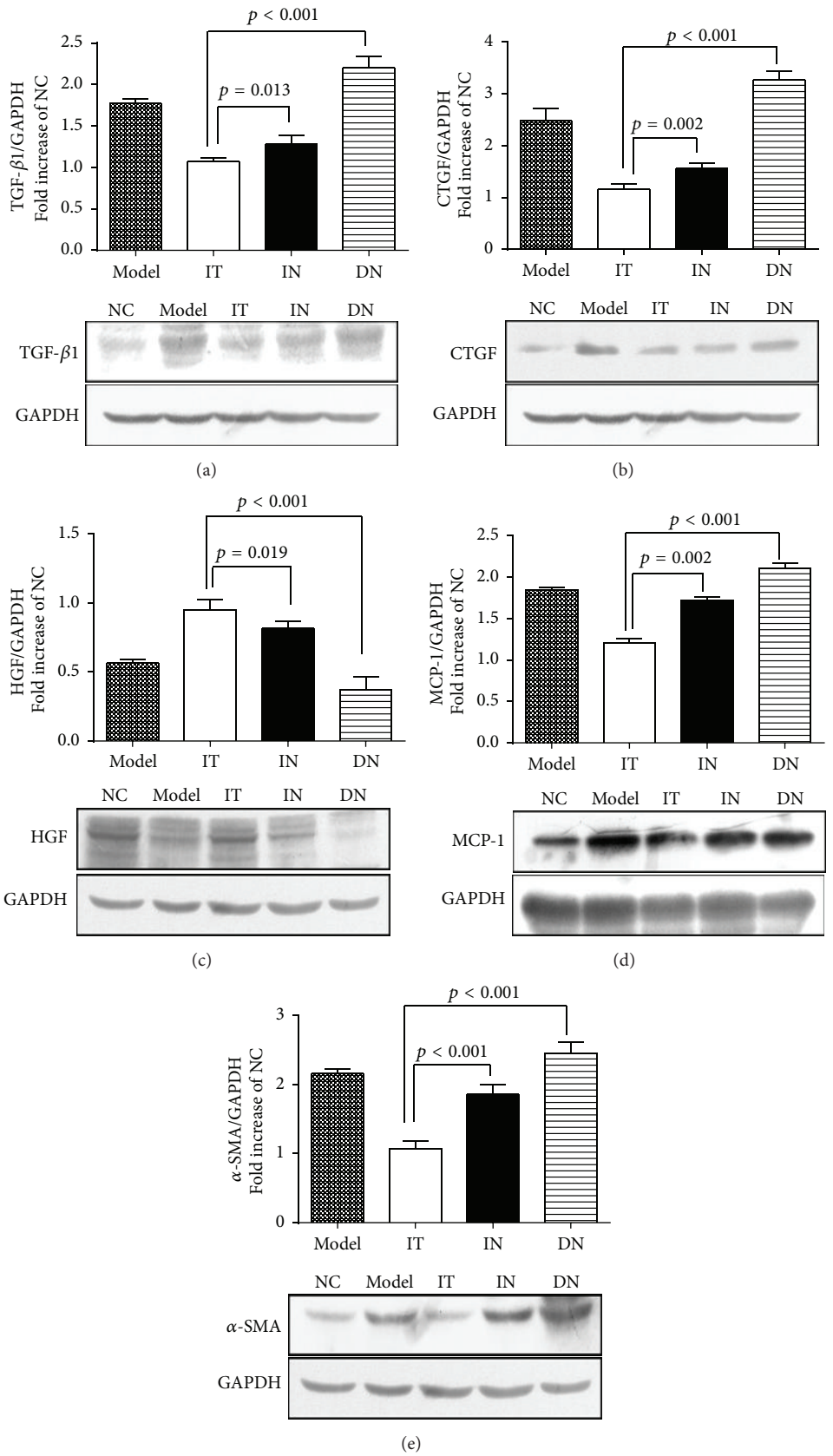

Figure 6: Western blotting analysis of fibrogenic and interstitial fibrosis factors in renal tissues. Western blotting analysis was performed to assess the protein expression of fibrogenic and interstitial fibrosis factors in the kidneys in each group. (a) TGF-β1. (b) CTGF. (c) HGF. (d) MCP-1. (e) α-SMA. Model group: diabetic nephropathy model rats established at 12 weeks.

intervention of renal fibrosis is extremely important. Studies have also indicated that the actions of fibrotic factors and cytokines, such as TGF-β1, HGF, CTGF, α-SMA, and MCP-1, play significant roles in the progression of renal fibrosis in early DN. Compared with the untreated DN rats, the expression of HGF was obviously elevated, accompanied by a significant decrease in the expression of other factors, after islet transplantation. TGF-β1 is regarded as the central fibrogenic factor in the pathogenesis of progressive renal fibrosis in DN. Its overexpression stimulates renal fibrosis by promoting podocyte and tubular epithelial and glomerular endothelial cell apoptosis, activating interstitial fibroblasts, and inducing the activation of α-SMA expression and mesangial cells to produce large amounts of ECM components [28]. HGF might inhibit TGF-β1 expression and prevent the progression of renal fibrosis in various animal models [29]. Components of the diabetic milieu also could promote the recruitment of macrophages by stimulating the expression of MCP-1 in the kidneys in response to renal injury [30]. The results of our study showed that renal fibrosis was improved significantly after islet transplantation compared with that observed after insulin therapy.

Because it is associated with a lower risk of surgery and fewer side effects than full-pancreas transplantation, pancreatic islet transplantation had been widely investigated as a promising strategy for treating diabetes mellitus and/or its complications. It is well known that sustained hyperglycemia is one of the major causes of DN. Regular oral insulin administration or insulin injection cannot always maintain plasma glucose within the normal range, resulting in fluctuations in the blood glucose level [31, 32]. The kidneys remain in a suboptimal state when hyperglycemia cannot be eliminated. However, because islet transplantation is capable of physiologic regulation *in vivo*, it can control the secretion of insulin automatically to maintain the blood glucose level within the normal range. Hypoglycemia is considered one of the most dangerous complications occurring in clinically diabetic patients, and intensive insulin therapy protocols have been widely used in the clinical intensive care of diabetes mellitus; notably, the incidence of hypoglycemia is markedly increased if the blood glucose level is not constantly monitored [33]. Nevertheless, because of the self-regulation of physiological systems, excessive insulin secretion does not occur after islet transplantation. During this study, although a rat's blood glucose level was not fully maintained within the normal range after transplantation, the renal damage and fibrosis of DN of this rat were still alleviated as observed in the late examination. These findings could indicate that transplantation of a small amount of transplanted islets also had ameliorative effects on DN, but further study is required for clarification.

Relevant signaling pathways about the ameliorative effects of islet transplantation have not been illuminated very clearly, and these topics also will be the main focuses of our next experiments. Recent studies have demonstrated that abnormalities in signaling pathways could also contribute to the pathologic signs of DN. An increasing number of studies have attempted to elucidate the molecular mechanisms of DN to facilitate the development of preventative strategies and effective therapies [34]. Fiorina and his colleagues have found the induction of immune-related protein B7-1 is associated with the progression of proteinuria in human and animal glomerular diseases. The expression of B7-1 in podocytes is upregulated in high glucose conditions, which can induce podocyte morphologic abnormalities and promote cell death. Targeting B7-1 has been suggested as a promising effective method on preventing cure diabetic nephropathy in their studies [35]. These new pathogenic mechanisms have been clarified by manipulation of gene expression in animal models, and the use of such models might contribute to the understanding and clinical therapy of DN. Due to the limitations of the present study, many important issues remain to be addressed, particularly the survival of the transplanted islets and the minimum equivalent required to treat diabetes mellitus. Meanwhile, research has indicated that C-peptide might play an important role in the control of blood glucose in diabetic patients. Johansson et al. [36] have found that the combined administration of insulin, proinsulin, and C-peptide has greater efficacy for the treatment of diabetes than insulin injection alone. Whether islet transplantation can regulate the secretion of C-peptide is also a vital issue [37]. The encouraging studies and results of the present study could provide a basis for the clinical application of islet transplantation.

In conclusion, our results demonstrated that islet transplantation could reverse various symptoms of early DN in a rat model, and the ameliorative effects on kidney injury and renal fibrosis were obviously better with islet transplantation than with insulin therapy. The recovery of impaired podocyte structure and function is the key to the treatment of diabetic nephropathy. In this study, islet transplantation under the kidney capsule significantly ameliorated the impaired renal function and microscopic damage in the early diabetic nephropathy rats. These findings may provide new possibilities to treat or prevent early DN and other complications in patients with diabetes mellitus in the future.

Competing Interests

All the authors declare that there are no financial or nonfinancial competing interests regarding the publication of this paper.

Acknowledgments

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