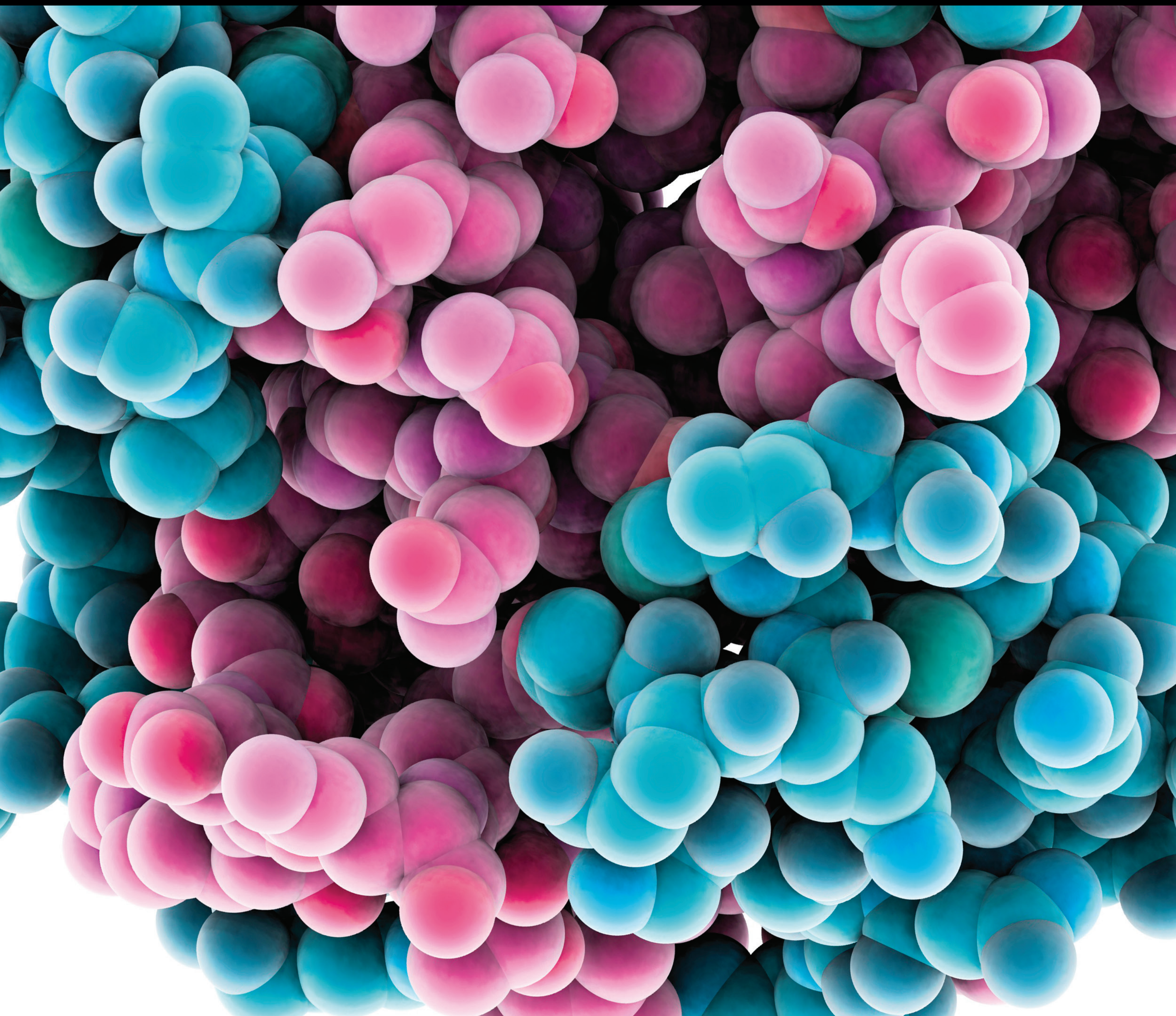
Journal of Diabetes Research

Diabetic Nephropathy: Proteinuria, Inflammation, and Fibrosis

Guest Editors: Shirong Zheng, David W. Powell, Feng Zheng, Phillip Kantharidis, and Luigi Gnudi



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Editorial

Diabetic Nephropathy: Proteinuria, Inflammation, and Fibrosis

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Diabetic nephropathy (DN) is a serious complication of diabetes; it initially manifests with microalbuminuria and progresses towards end-stage renal failure. Sustained diabetes-related metabolic and haemodynamic perturbations can induce subclinical low-grade renal inflammation and drive kidney from repair response to damage process, eventually to renal fibrosis. In this special issue, we include articles regarding inflammation, Chinese herbs, and systems biology to present up-to-date information on immune cells, chemokine receptor, and biomarkers in DN, displaying combined therapy in treatment of DN and highlighting the effective approach in exploring genetic susceptibility of DN.

(1) Inflammation. Despite the broad themes covered by this special issue, all articles focus on a common theme: immune cells and inflammation. Hyperglycemia and oxidative stress, as well as albuminuria per se, can lead the immune and inflammatory cells to infiltrate into kidney and release proinflammatory cytokines. This inflammatory "repair process" reverts to and manifests as a "chronic unfavorable process" that eventually leads to the disease phenotype (renal fibrosis). The review article by Z. Zheng and F. Zheng summarizes the role of immune cells and inflammation in DN, highlighting the contribution of APC cells, T-helper cells, and tubular epithelial cells to the inflammation. S. Zheng et al. reported the renal expression of decoy chemokine receptor ACKR2 in DN patients and renal protection in diabetic mice with ACKR2 gene knockout, revealing the unexpected negative role of ACKR2 in diabetic kidney disease. Association of haemostatic and inflammatory biomarkers with nephropathy in type I diabetic patients is shown by C. P. Domingueti et al., indicating potential therapeutic targets for DN.

(2) Chinese Medicine. Herbs are major form of therapy in traditional Chinese medicine. Their value has been illustrated by the discovery of artemisinin [1], a drug saving millions of lives from malaria, derived from an active ingredient of Chinese herb. Chinese medicine is part of the treatment used against DN in China. In review by G. Sun et al., over 20 recipes of herb medicine and 30 single herbs or monomers are summarized. These therapies have showed efficacy at improving albuminuria and inflammation in diabetic patients. Ongoing research programs focus on identifying the effective component(s) contained in the most promising herbs with the ultimate aim of developing safe and novel compounds for the treatment of DN.

(3) Systems Biology. As defined by NIH, systems biology is an approach used in biomedical research to understand the "bigger picture"—be it at the level of the organism,

tissue, or cell—to reconstruct the biology from huge volumes data using computational and mathematical methods. This is in stark contrast to decades of reductionist biology, which involved taking the pieces apart in order to understand the biology [2]. As technology advances, genomics, proteomics, and metabolomics become reliable, affordable, and readily available to explore the molecular profiles of human disease. F. Conserva et al. present a systems biology overview of human DN, from genetic susceptibility to posttranscriptional and posttranslational modifications. Molecules identified by genomics, transcriptome, and epigenetic studies in area of DN await to be validated. Using proteomics approach, M. Barati et al. report the influence of acute high glucose exposure on the change in protein abundance in murine glomerular mesangial cells. These discovery-based studies shed new light and new perspectives in DN research.

Acknowledgments

Our thanks go to all the authors and reviewers. As guest editors, we are honored to share this discussion with you.

Shirong Zheng
David W. Powell
Feng Zheng
Phillip Kantharidis
Luigi Gnudi

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Research Article

Influence of Acute High Glucose on Protein Abundance Changes in Murine Glomerular Mesangial Cells

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The effects of acute exposure to high glucose levels as experienced by glomerular mesangial cells in postprandial conditions and states such as in prediabetes were investigated using proteomic methods. Two-dimensional gel electrophoresis and matrix assisted laser desorption ionization time of flight mass spectrometry methods were used to identify protein expression patterns in immortalized rat mesangial cells altered by 2 h high glucose (HG) growth conditions as compared to isoosmotic/normal glucose control (NG*) conditions. Unique protein expression changes at 2 h HG treatment were measured for 51 protein spots. These proteins could be broadly grouped into two categories: (1) proteins involved in cell survival/cell signaling and (2) proteins involved in stress response. Immunoblot experiments for a protein belonging to both categories, prohibitin (PHB), supported a trend for increased total expression as well as significant increases in an acidic PHB isoform. Additional studies confirmed the regulation of proteasomal subunit alpha-type 2 and the endoplasmic reticulum chaperone and oxidoreductase PDI (protein disulfide isomerase), suggesting altered ER protein folding capacity and proteasomal function in response to acute HG. We conclude that short term high glucose induces subtle changes in protein abundances suggesting posttranslational modifications and regulation of pathways involved in proteostasis.

1. Introduction

Renal glomerular mesangial cells (GMCs) functions are altered in diabetic nephropathy by chronic exposure to high glucose (HG) or exposure to glycated albumin [1–4]. The early effects of hyperglycemia are thought to be dominated by hemodynamic factors including glomerular hyperfiltration and shear stress leading to damage by microalbuminuria or proteinuria [5–10]. The early histopathology of diabetic nephropathy is characterized by a thickening of the glomerular basement membrane (GBM) and an accumulation of extracellular matrix (ECM) in the glomerular mesangium. The damaging effects of chronic hyperglycemia on various kidney glomerular cell types such as mesangial cells, podocytes, and endothelial cells have been intensely studied. The theories that have been addressed include increased substrate channeling into the polyol pathway and the hexosamine pathways and increased production of reactive oxygen species (ROS) and activation of protein kinase C (via advanced glycation end-products (AGE), diacylglycerols (DAG), and/or reactive oxygen species (ROS)) [11–13]. These advances in our understanding of the effects of chronic hyperglycemia on renal physiology have not been matched by understanding of the effects of acute (2 h) hyperglycemic conditions episodically experienced by cells like the GMC in states such as prediabetes. We hypothesize that understanding these acute changes induced by hyperglycemia might yield insight into the mechanisms through which chronic hyperglycemia disrupts mechanisms used to maintain normal glomerular function.

2. Material and Methods

2.1. Cell Culture. The rat GMC line CRL-2573 (ATCC) maintained normal growth media (DMEM: 5 mM D-glucose, 15% FBS) under 5% CO_2 at 37°C. The cells (passages 10–15) were plated in Corning T25 flasks and cultured until 70–80% confluence was reached. Normal media were removed from cells and replaced with DMEM supplemented with 0.5% FBS/5 mM D-glucose. After 24 h, media were removed and replaced with isoosmotic-normal glucose (NG^*) media (DMEM-5 mM D-glucose, 20 mM mannitol, and 0.5% FBS) or high glucose (HG) media (DMEM: 25 mM D-glucose, 0 mM mannitol, and 0.5% FBS), for 2 h. For 2DE analysis, after 2 h treatment, the total protein was collected as previously described [14] using IPG rehydration buffer supplemented with protease inhibitors.

2.2. Cell Viability. Cell viability was determined after 2 h HG and NG^* treatment using the MTT assay [15] as described by the manufacturer (Sigma, St. Louis, MO, USA).

2.3. Two-Dimensional Electrophoresis (2DE) and Image Acquisition. 2DE experiments were conducted as reported previously [14]. Murine GMC protein (75 μg) was rehydrated overnight into IPG (pH 3–10; 7 cm; Invitrogen) strips. The strip was focused for a total of 1200–1300 Vh with a final 30 min focusing period at 2000 V constant. Proteins were separated in the second dimension on 4–12% Bis-Tris mini gels (8 cm × 8 cm). The gel slabs were fixed in 10% methanol and 7% acetic acid and then transferred to SYPRO-Ruby protein gel stain (Molecular Probes, Oregon, USA) for 18 hours. Gels were scanned using a PerkinElmer ProXpress CCD-based digital imager at 50 μm resolution. The gel/stain exposure and emission acquisition times were varied to maximize the detector response while avoiding detector saturation. The image files were matched, reference gels were created, and spot volumes were determined using Progenesis Discovery software (Nonlinear Dynamics, Newcastle upon Tyne, UK). A student's t-test is used to evaluate all matched spot pairs. Protein spots that were found to have variable spot volumes between samples were statistically compared by spot mean and SEM.

2.4. Proteomic Analyses. Protein gel spots were digested as previously described [14]. MALDI-TOF and TOF/TOF MS data were acquired on the tryptic digests using an AB4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) and analyzed using Matrix Science Mascot (ver. 2.0) as described previously [16]. Data was analyzed assuming (a) monoisotopic peptides masses, (b) cysteine carbamidomethylation, (c) variable oxidation of methionine, (d) maximum of one missed trypsin cleavage, and (e) a mass accuracy of greater than 150 ppm for MS data and 0.3 Da for MS-MS data against the SwissProt (release 52.0, 20070307) protein database (261513 sequences; 95638062 residues) constrained to the mammalian (50870 sequences) taxa. Limitation of the original protein mass was not employed within the Mascot search. Protein identifications were accepted for protein identifications that include using MASCOT MS + MS/MS analysis with significant MOWSE scores ($p < 0.05$; for MS MOWSE score of 60 which equals significance and for MS/MS MOWSE peptide ion score alone of 40 which equals significance).

2.5. Confocal Microscopy. Confocal microscopy images were obtained as previously described [17]. Briefly, multichambered cover glass wells (Nunc, Naperville, CT) were seeded with GMC cells. Cells were serum starved with 0.5% FBS-NG medium 24 h before 2 h glucose treatment. Cells were rinsed three times with PBS that contained calcium and magnesium and fixed in 3.7% paraformaldehyde in PBS for 10 min, followed by permeabilization with 0.025% NP-40 in PBS for 15 min. Cells were incubated with primary antibody (1 : 250 anti-PHB in PBS/0.025% NP-40) at 20°C, rinsed five times with PBS/0.025% NP-40, and incubated with the Alexa Fluor 488 conjugated secondary antibody (1 : 1000) at 20°C. The cells were rinsed five times with PBS/0.025% NP-40, incubated with 300 nM DAPI for 5 min, and rinsed three times with PBS. Images were acquired using a Zeiss confocal microscope and analyzed using LSM510 software. Z scan analysis was performed by scanning at 1 μm intervals and three-dimensional reconstruction of the fluorescence images. The images for PHB and for DAPI were merged in a single image to elucidate PHB cellular distribution. Fluorescence intensity measurements (mean fluorescence intensity per μm^2) were computed per cell (n = 4-5 cells per treatment replicate per treatment condition) and used to estimate differences in PHB nuclear and cytoplasmic distribution.

2.6. Protein Immunoblotting (IB). 1DE and 2DE protein immunoblots (IB) were conducted as previously described [14]. Total cell lysate samples were separated by 2DE ($n = 3$ HG, $n = 3$ NG^*). For 2DE IB analysis, following IEF of mesangial proteins, the plastic backing of the IPG strips was trimmed off. The acidic most point of the strip was aligned in the IPG well of the Bis-Tris mini gels adjacent to the MW standard lane of the minigel. This procedure insured uniform alignment of IPG strips to the MW standards, in order to compare PHB migration pattern between experimental conditions. Following 1DE or 2DE electrophoresis and transfer, membranes were immunoblotted for PHB (Santa Cruz Biotechnologies, Santa Cruz, CA) at a 1 : 1000 dilution in 5% albumin in Tris-Tween-20 buffered saline (TTBS). PHB spots were imaged on film with luminol images aligned and quantified by densitometry analysis comparing the means of the acidic third and basic third of the PHB charge trains to the total train densitometry. Additional antibodies used for 1DE immunoblots were anti PDI (Stressgen; San Diego, CA) at a concentration of 1 : 10,000 and PSMA2 (Cell Signaling; Danvers, MA) at a concentration of 1 : 1000.

2.7. Analysis of Protein Expressional Networks. Ingenuity Pathways Analysis bioinformatic tool (Ingenuity Systems, Mountain View, CA) uses a curated database (Ingenuity Systems Knowledge Base) of previously published findings on mammalian biology from the public literature to evaluate proteins lists inclusive of expression ratios for protein

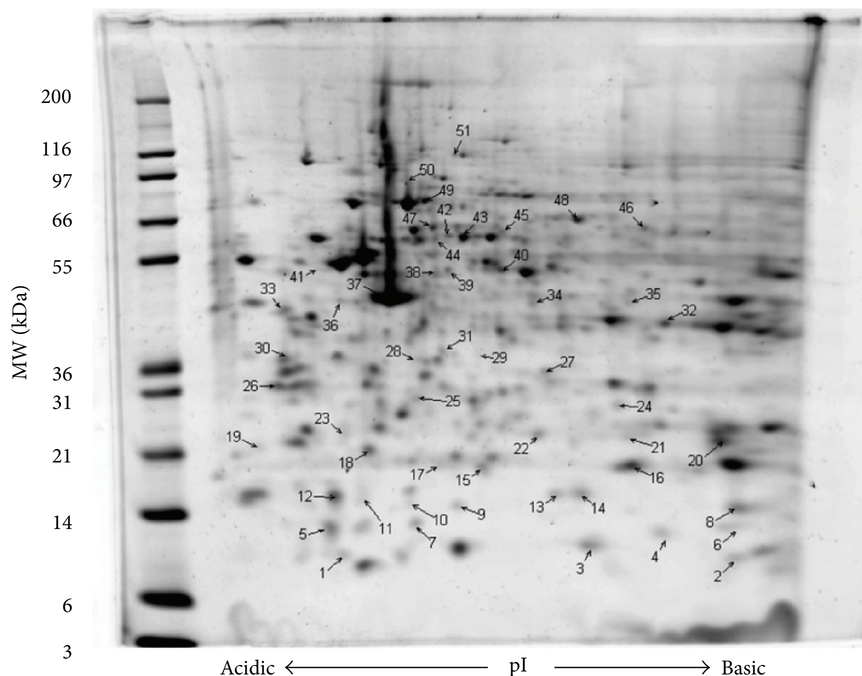

Figure 1: Murine GMC proteome altered by acute (2 h) exposure to HG culture conditions. GMC cells were grown to 80% confluence and were serum-starved (0.5% FBS) overnight, and were treated for 2 h with 25 mM glucose (HG) or 5 mM glucose + 20 mM mannitol (NG*) as an isoosmotic control. Cells were lysed using 2DE buffer and 75 μg protein used for 2DE analysis. Proteins whose expression is altered by 2 h HG are annotated on the gel with identifications provided in Table 1. Data are representative of five individual gels for HG and for NG* conditions.

expressional patterns. The purpose of the evaluation is to establish within the lists of provided expressional data relational networks of protein interactions (e.g., direct protein-protein interaction and transcriptional control). Analysis of submitted protein lists with expressional ratios using the Ingenuity knowledge base was used to identify direct interactions between mammalian orthologs.

Murine GMC proteins demonstrating statistically significant expression between 2 h HG and 2 h NG* as well were analyzed by the Ingenuity Knowledge Base and Pathways Analysis tool. The data output identifies nodes characterizing individual proteins and edges characterizing biological relationships. Putative protein networks are rank ordered according to p value ($-\log_{10}{}^{p}$), where the p value is a measure of random association of the listed proteins.

2.8. Statistical Analysis. Statistical analysis of relative spot pixel intensity from 2D gels ($n = 5$, 2 h each group) and analysis of PHB, PDI, or PSMA2 for HG versus NG* expression by IB was performed using two-tailed, unpaired t-test. p values < 0.05 were considered significant.

3. Results

3.1. Alteration of Protein Expression by Acute High Glucose. Based on the MTT assay results (data not shown), GMC viability did not statistically vary between 2 h HG and NG* treatments. To determine proteins regulated by 2 h HG treatment, protein spot volume lists were curated by first estimating intergel variability in matched protein spot volumes (averaged CV for 20 matched spots = 0.17). Next, all intraglucose treatment matched gel spot volumes having a CV greater than 0.35 (2 × CV) were discarded. Fifty-one (51) protein spots had a spot volume CV of less than 0.35 and uncorrected t-test values of ≤0.05. Thirty-five protein spots had increased expression and 16 protein spots had decreased expression with 2 h HG treatment and all were analyzed using proteomic methods based on MASCOT MOWSE scoring including MALDI TOF/TOF peptide fragmentation (sequence tagging) data with a significance p value ≤ 0.05 for all the reported protein identities. A representative 2DE gel image (with annotations) and tabulated information for 51 regulated protein spots are provided (Figure 1; Table 1). In general, all proteins identified were observed migrating in the gels at the correct molecular weight plus or minus 10% except for gel spot 5. Cofilin-1 was identified migrating at a molecular weight of approximately 9000 Da and a pI of 5.3. Cofilin-1 nominally has a translated molecular weight of 18,749 Da and a pI of 8.2. Two additional cofilin-1 containing gel spots as well as one HSP10 containing gel spot were observed to focus on isoelectric points less than 0.5 pH units, more acidic than expected. Twenty-three proteins were observed to focus on isoelectric points greater than 0.5 pH units, more basic than expected. The remaining gel spots identified proteins within 0.5 pH units of the expected pI.

3.2. Analysis of Protein Expressional Networks. Bioinformatic analysis of protein expression in 2 h NG* versus 2 h HG

Table 1

Spot	Protein name	Gene product	(HG/NG*)	Theoretical M_r	Theoretical pI	Observed M_r	Observed pI	IPA network	Percent coverage
1	Not identified		1.23						
2	10 kDa heat-shock protein, mitochondrial	CH10_RAT	2.37	10895	8.9	8000	8.3	n/a	77
3	Calpactin I light chain	S10AA_RAT	1.37	11182	6.3	8000	7.4	1	45
4	Macrophage migration inhibitory factor	MIF_RAT	1.34	12640	6.8	9000	7.8	3	26
5	Cofilin-1	COF1_RAT	1.5	18749	8.2	9000	5.3	n/a	34
6	Not identified		1.70						
7	Not identified		1.24						
8	Profilin-1	PROF1_RAT	1.34	15119	8.5	15000	8.4	1	45
9	Cystatin B	CYTB_RAT	0.82	11303	5.9	10000	6.5	2	80
10	Not identified		0.66						
11	Coactosin-like protein	COTL1_MOUSE	1.74	16048	5.3	16000	5.5	1, 2	37
12	Galectin-1	LEG1_RAT	1.23	15189	5.1	15000	5.3	3	61
13	Histidine triad nucleotide-binding protein 1	HINT1_MOUSE	1.39	13882	6.4	11000	7.3	3	49
14	40S ribosomal protein S12	RS12_RAT	1.34	14858	6.8	15000	7.2	2	44
15	Nucleoside diphosphate kinase A (NDK A)	NDKA_RAT	1.68	17296	6.0	16000	6.6	3	39
16	Nucleoside diphosphate kinase B	NDKB_RAT	1.26	17386	6.9	18000	7.6	1	66
17	Not identified		1.89						
18	Eukaryotic translation initiation factor 5A	IF5A1_RAT	1.23	17049	5.1	20000	5.6	2	43
	MIR-interacting saposin-like protein	MSAP_MOUSE		21096	5.0	20000	5.6	n/a	29
19	Not identified		1.64						
20	Cofilin-1	COF1_RAT	1.63	18749	8.2	21000	8	1	65
21	Cofilin-1	COF1_RAT	1.59	18749	8.2	21000	7.6	1	60
22	Cofilin-1	COF1_RAT	1.57	18749	8.2	21000	7	1	54
23	Not identified		1.61						
24	Proteasome subunit alpha type 1	PSA2_RAT	1.54	26024	6.9	23000	7.5	1	47
25	Heat-shock protein beta-1	HSPB1_RAT	2.31	22936	6.1	23000	6.1	1	39
	Phosphoserine phosphatase	SERB_RAT		25180	5.5	23000	6.1	n/a	33
26	14-3-3 protein epsilon	1433E_RAT	1.37	29326	4.6	30000	4.8	1	29
27	Proteasome subunit alpha type 2	PSA1_RAT	1.78	29784	6.2	31000	7.2	1	36
28	Prohibitin	PHB_RAT	2.24	29859	5.6	32000	6.1	2	62
29	Not identified		2.33						
30	Proliferating cell nuclear antigen	PCNA_RAT	1.46	29072	4.6	34000	4.9	1	31
31	Heat-shock protein beta-1	HSPB1_MOUSE	0.70	23057	6.1	35000	6.3	n/a	25
32	Annexin A2	ANXA2_RAT	0.83	38939	7.6	40000	7.8	1	56
33	Reticulocalbin 3 precursor	RCN3_HUMAN	1.80	37470	4.7	41000	4.9	2	20
34	Macrophage capping protein	CAPG_RAT	1.53	39060	6.1	41000	6.9	3	20
35	Acetyl-CoA acetyltransferase, cytosolic	THIC_RAT	2.15	41538	6.9	41000	7.7	3	28
36	SUMO-activating enzyme subunit 1	SAE1_RAT	2.15	38945	5.0	41000	5.4	2	54
37	Actin, cytoplasmic-1 (beta-actin)	ACTB_RAT	0.58	42052	5.3	42000	5.7	1	30
	Actin, cytoplasmic-2 (gamma-actin)	ACTG_RAT		42108	5.3	42000	5.7	1	30
38	Not identified		0.6						
39	Actin-like protein 3	ARP3_MOUSE	0.53	47783	5.6	50000	6.5	1	38
40	Enolase 1	ENOA_RAT	0.60	47440	6.2	52000	6.7	1	38
	RAB GDP dissociation inhibitor beta	GDIB_RAT		51018	5.9	52000	6.7	n/a	34
41	Not identified		0.37						
42	Not identified		0.71						
43	Protein disulfide-isomerase A3 (ERp57)	PDIA3_RAT	0.78	57044	5.9	58000	6.4	3	50
44	GRP58	HNRPK_RAT	0.68	51230	5.4	57000	6.3	1	31
45	Not identified		2.21						
46	Heterogeneous nuclear ribonucleoprotein L	HNRPL_MOUSE	1.40	60712	6.7	58000	7.8	2	25
47	T-complex protein 1, epsilon subunit	TCPE_RAT	0.78	59955	5.5	58000	6.3	1	34

Table 1: Continued.

Spot	Protein name	Gene product	(HG/NG*)	Theoretical M_r	Theoretical pI	Observed M_r	Observed pI	IPA network	Percent coverage
48	Hsc70/Hsp90-organizing protein	STIP1_RAT	1.16	63158	6.4	65000	7.2	1	38
49	GRP 75	GRP75_RAT	0.82	74097	6.0	75000	6.1	1	31
50	Not identified		1.35						
51	RAB 6 interacting protein 2 (ERC protein 1)	GANAB_MOUSE	0.68	107300	5.7	116000	6.4	2	21

Murine GMC protein expression at 2 h culture HG versus 2 h culture NG*.

was achieved using the Ingenuity Knowledge Base and Pathways Analysis tools. The top three canonical pathways determined to be activated from 2 h acute high glucose exposure were actin-based motility by Rho, RhoA signaling, and the protein ubiquitination pathway. Analysis of protein expressional networks from murine GMC 2 h NG* and 2 h HG protein expressional data suggested three primary expression networks. Network 1 (score 49) addressed cancer, reproductive system disease, and hematological disease and included 25 identified proteins out of 35 total network components (Figure 2(a)). Network 2 (score 19) addressed cell death and survival, drug metabolism, and lipid metabolism and included 9 identified proteins out of 35 protein nodes (Figure 2(b)). Network 3 (score 14) addressed cellular movement, cellular compromise, cellular function, and maintenance and was composed of 7 identified proteins out of 29 possible network proteins. Prominent nodes within Network 1 were centered on signaling proteins including proteins involved with ubiquitination, cyclin D, ERK1/ERK2 MAP-Kinase, HSP90, ROCK, and histones h3 and h4. Prominent nodes in network 2 were centered on the VEGF, TNF, TGFβ1, tumor protein 53 (TP53), and ubiquitination.

3.3. Immunochemical Analysis for the Effect of High Glucose on the Expression of Proteins. Immunoblot (IB) analyses of the selected proteins were used to confirm the 2DE findings. Prohibitin (PHB) was selected for confirmation as it was one of the most strongly regulated protein spots and was also a component of IPA Network 2 with direct interaction with a prominent network node of TNF. The expression by 1DE (Figures 3(a) and 3(b)) supported a trend in increased total PHB abundance, but 2DE IB analysis of 2 h GMC cells cultured in HG and NG* showed a HG responsive and statistically significant ($p < 0.02$) increase in the acidic end of the PHB charge train (Figure 4). Confocal microscopy (Figures 5(a) and 5(b)) suggested that high glucose resulted in a statistically significant (p value < 0.0001) increased fractional abundance of PHB in the nucleus of the GMC.

Based on bioinformatics analysis defining regulation of protein ubiquitination pathways in one of the top three canonical pathways regulated, as well as a prominent node in protein expression Networks 1 and 2, we next analyzed the expression of proteins involved in protein homeostasis and found them to be regulated by 2DE analysis. Proteasome subunit alpha-type 2 (PSMA2) was confirmed to be increased in mesangial cells exposed to high glucose concentrations for 2 h (Figure 6). Comparative 2DE analysis also defined decreased expression of ER chaperone proteins such as PDI and GRP58, which may lead to increased unfolded protein load in mesangial cells and induction of proteasomal degradation processes. Immunoblot analysis of mesangial proteins for PDI confirmed 2DE findings of decreased expression of PDI (Figure 6).

4. Discussion

GMCs participate in glomerular growth and differentiation as well as in regulation of glomerular blood flow [3, 4]. It is well established that chronic hyperglycemia such as in an uncontrolled diabetic state detrimentally affects the renal glomerulus and produces a pathologic GMC phenotype [18–22]. On the other hand, a gap in knowledge exists for changes in GMC function and protein expression patterns which occur in individuals who experience longer postprandial elevated plasma glucose levels [23]. Therefore to ascertain the effects of short term high glucose conditions encountered by GMC in sub-pathologic/prediabetic states, we conducted proteomic studies comparing mesangial protein expression after 2 h HG conditions against mesangial protein expression after 2 h NG* growth conditions. The analysis of cell viability at 2 h in the treatment conditions determined that mesangial cell viability was not decreased by the treatment conditions and time. The protein expression differences observed between the growth conditions were not therefore attributed to variable degrees of cell proliferation. Expressional regulation of 51 identified protein spots were observed under the conditions of 2 h HG. These proteins can be grouped as follows: cytoskeletal proteins, calcium/phospholipid binding proteins, chaperones, and proliferation and signaling-related proteins.

Increased glucose levels are known to stimulate a variety of responses within GMC including remodeling of cytoskeletal elements like actin and actin binding proteins [24]. Two upregulated spots were identified as cofilin-1 and cofilin-2 and demonstrated a 55–60% increased expression. Cofilins are actin binding proteins that affect the mobility of actin monomers at the ends of actively growing actin filaments and increase actin filament turnover. Cofilins bind and sever the pointed actin ends and increase the actin monomer pool. During conditions of stress, cofilins participate in the nuclear import of actin [24]. Two protein spots demonstrating reduced expression by HG, identified as the actin capping proteins, F-actin capping protein β-subunit, and actin-like protein 3. Each of these proteins migrated at the expected M_r and pI. These proteins, respectively, demonstrated a 30% and 50% decreased presence in the 2DE gels.

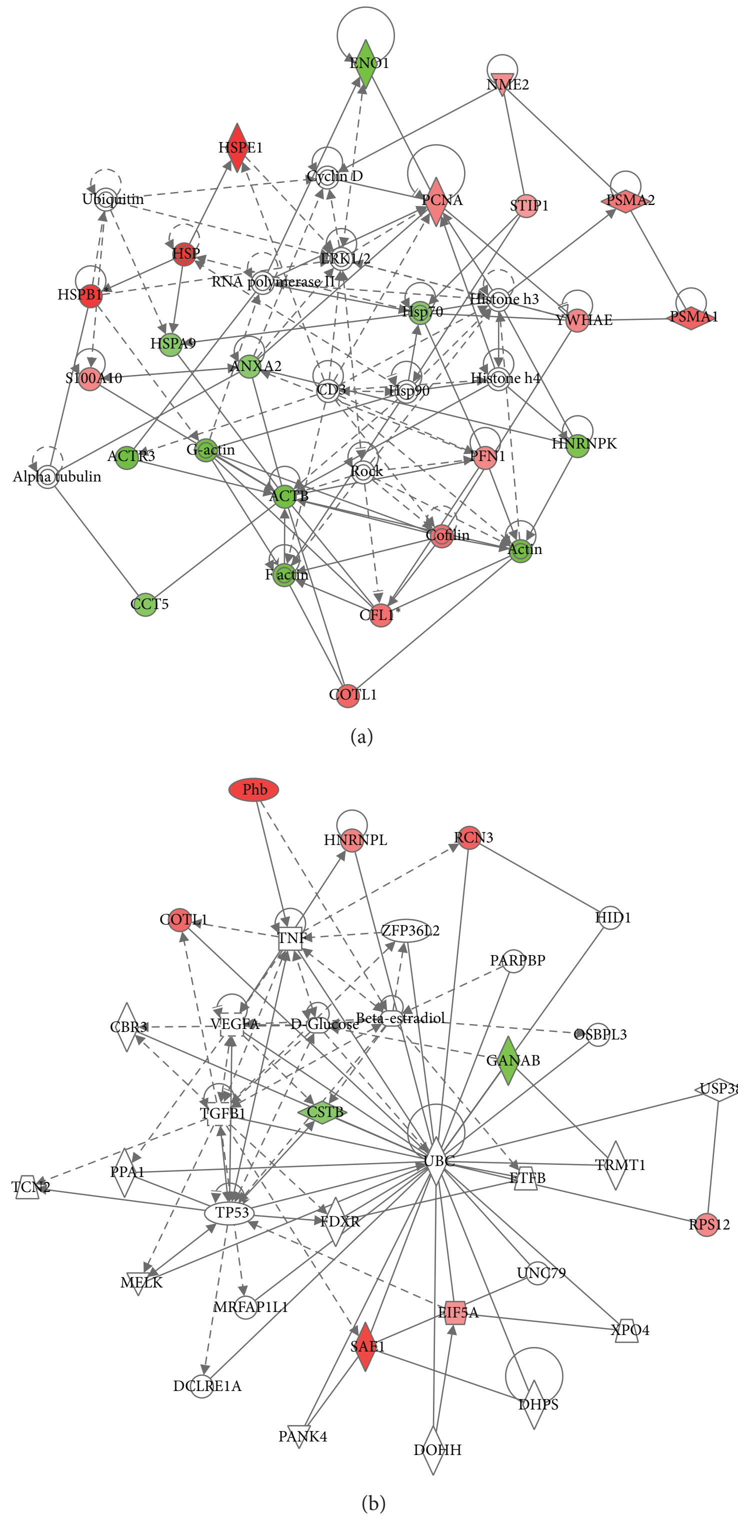

Figure 2: Network analysis of protein expression patterns using Ingenuity Pathways Analysis. (a) The top scoring network (Network 1) addressed cancer, reproductive system disease, and hematological disease and included 25 identified proteins out of 35 total network components. The score 49 suggests the odds of 1 out of 10^{49} for assembling randomly these protein identifications out of the existing murine protein database. (b) Network 2, defined by IPA, includes PHB. For (a) and (b), red indicates protein spots whose spot volume increased with 2 h high glucose. Green indicates proteins spots whose spot volume decreased with 2 h high glucose.

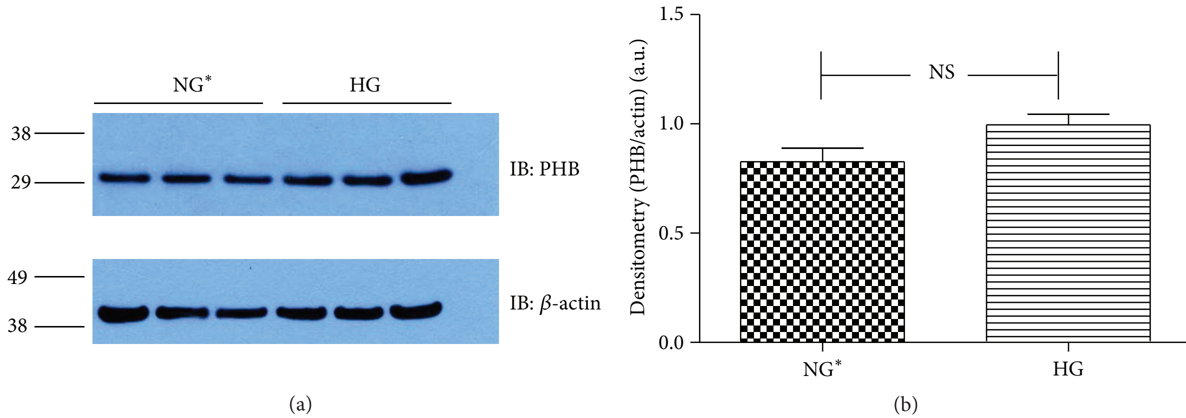

FIGURE 3: Validation of 2DE results for the enhanced PHB expression. Murine GMCs were cultured and treated for 2 h with HG and NG* as described. Cells were lysed in 2DE buffer, diluted into Laemmli buffer, and used for immunoblot experiments (a) and quantification of 1DE IB experiments for PHB expression normalized to total actin expression (b). Data is presented as a mean of three experiments. Statistical analysis of differences between the means of HG and NG* was achieved by t-test.

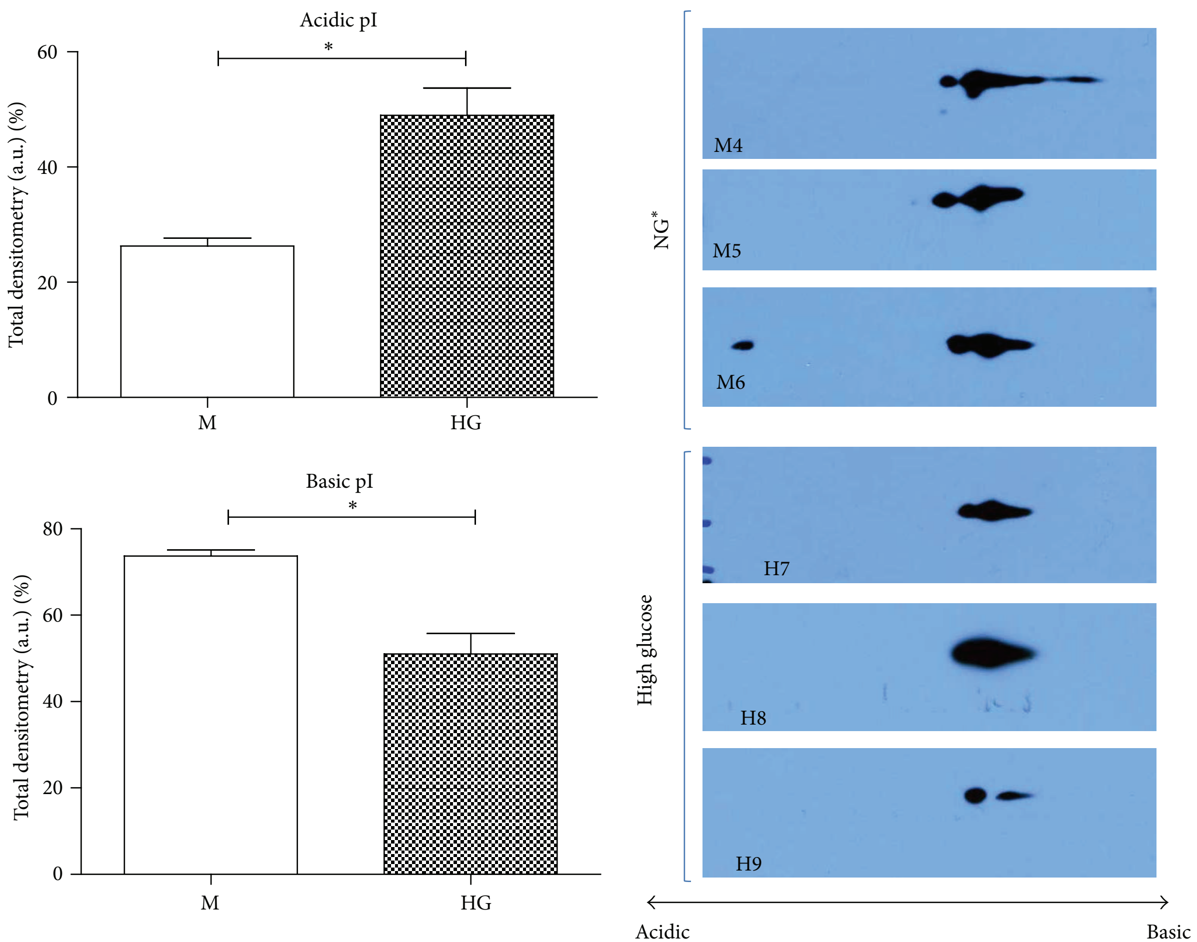

FIGURE 4: 2DE immunoblot experiments were used to determine the effects of HG and NG* on PHB isoforms. Following the transfer and development of PHB IB, images were aligned and densitometric measurements were estimated using ImageJ for the acidic one-third of the PHB charge train and for the basic two-thirds of the PHB charge train (IB images on right). Fractional values for PHB charge train components (acid and basic ends) were used to determine statistical significance differences (left bar graphs). M is the same as NG* (5 mM D-glucose + mannitol); HG is 25 mM D-glucose. $^*p$ value < 0.01.

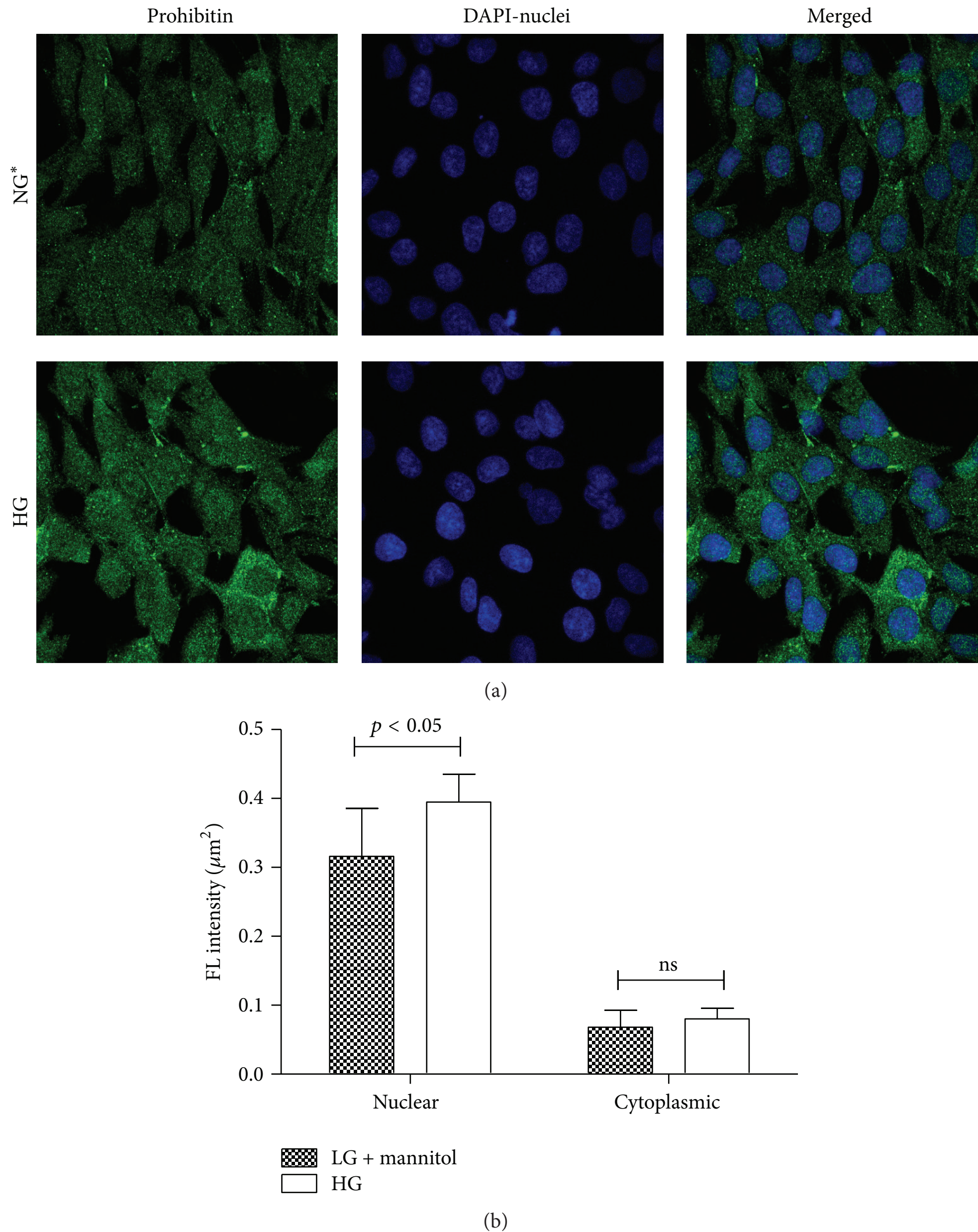

Figure 5: (a) Murine GMCs were seeded into 8-well chambered cover glass, grown, and treated as described in the methods. PHB detection was with the same primary antibody as used for IB. PHB detection with an Alexa Fluor 488 conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). Confocal software was used to estimate pixel density in GMC and in nuclei (as defined by DAPI). Nuclei pixel density was subtracted from total density and plotted (b). Differences were estimated by t-test with significance at p value < 0.05.

Additionally, an acidic isoform of the intermediate filament protein vimentin was downregulated.

Calpactin light chain (also referred to as S100A10 or p11) functions as a ligand of annexin II (annexin $II_2 : p11_2$) [25–27]. Calpactin and annexin II were shown here to be upregulated by approximately 37% and 23%, respectively by acute hyperglycemic conditions. Calpactin complexed to annexin II is known to interact with the C-terminus of cytosolic phospholipase A2 and inhibits cPLA2 activity thus reducing inflammatory responses from the release of arachidonic acid [28]. Upregulation of reticulocalbindin 3 is necessary for increased sequestration of Ca^{2+}. The increased Ca^{2+} is in turn needed by other proteins found in the reticuloplasm like GRP78 or PDIA3 [29]. These observations of regulated changes in actin cytoskeletal protein and calcium binding protein expression, when taken together, are consistent with the known responses of mesangial cells to HG under more chronic conditions [30, 31].

Molecular chaperones have been well described in the literature as protein quality control managers that assist with the maintenance of cellular function in the face of stress conditions like heat stress, osmotic stress, or oxidant

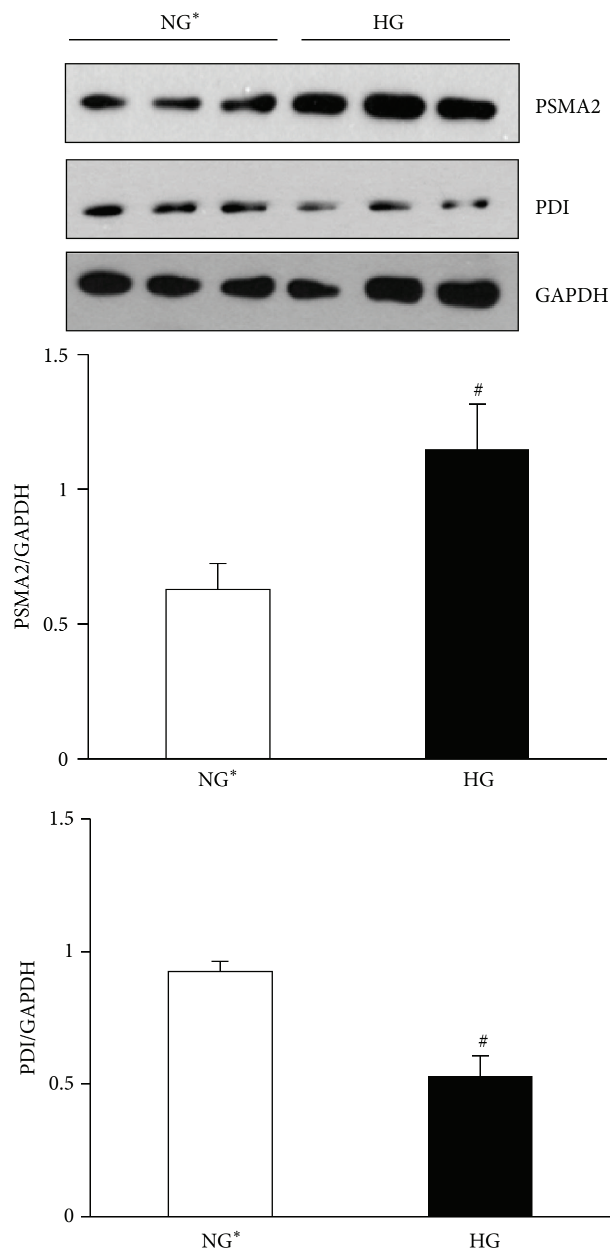

Figure 6: Correlative validation of 2DE results for the regulation of pathways involved in proteostasis. Immunoblot analysis of PSMA2 and PDI from mesangial cells cultured for 2 h in HG or NG* medium. Expression of PSMA2 increased whereas PDI decreased following 2 h HG. Bar graphs, densitometric quantitation of PSMA2 or PDI normalized to GAPDH for each lane. Data are average ± SEM $^{\#}p < 0.05$ versus NG*.

stress. Specific chaperones are spatially organized throughout the cell via organellar localization [32, 33]. The bulk of all mitochondrial proteins are synthesized under the direction of cell nuclear transcripts in the cytoplasm [34]. High molecular weight proteins are trafficked through and between the mitochondrial membranes and into mitochondrial matrix and require protein folding chaperone such as PHB and HSP10 for efficient protein folding [35]. A protein spot containing PHB, possibly a posttranslationally modified form causing an acidic shift in PHB pI, was found to exhibit expressional regulation by 2DE, 2DE IB and increased nuclear localization by confocal microscopy analysis, following acute (2 h) glucose exposure in GMCs. PHB has been reported to exist as a membrane resident chaperone that participates in the protein folding pathway of mitochondrial-derived integral membrane proteins like COX2p and COX3p. Moreover, movement of PHB between the mitochondria and nucleus has been shown to play an important role in signaling mitochondrial oxidant stress and regulating apoptosis and transcription during stress, highlighting the importance of this protein to mitochondrial-nuclear communication [36, 37]. In the current study, observations of increased acidic forms of PHB, increased PHB nuclear localization, increased HSP10, and decreased GRP75 at 2 h HG stimulation suggest that acute hyperglycemic conditions may promote protein-structural stress within the mitochondrial matrix promoting translocation of PHB to the nucleus for an as-of-yet determined reason in GMCs. In addition, bioinformatic analysis grouped PHB and additional proteins regulated by 2 h HG in a network including mediators known to be involved in the pathogenesis of diabetic nephropathy and fibrosis, such as TGFβ, VEGF, and TNF [1, 38], highlighting a potentially novel role for PHB in GMC responses to HG.

One aspect of cell cycle control is polyubiquitination of cytoplasmic or nuclear proteins [39]. Polyubiquitination is a trigger for the trafficking of the modified protein to the proteasome for degradation. A second aspect of cell cycle control is exercised through monoubiquitination of nuclear proteins like histones [40–42]. Our observations with increased expression of PSMA2 are specific to acute exposure of cells to medium containing high glucose as compared to isoosmotic low glucose medium and suggest the likelihood of increased proteasomal activity. These findings are in part supported by the observations of decreased ubiquitinated cytosolic proteins in mesangial cells with 2 h high glucose concentrations (data not shown). Together, increased expression of PSMA2 and decreased expression of PDI with acute exposure to high glucose concentrations suggest regulation of pathways involved in proteostasis and/or cell stress response. In the ER, PDI serves an oxidoreductase chaperone regulating disulfide bonds [43] and its activity is decreased in liver cells of diabetic mice [44]. Furthermore, kidneys and liver of diabetic mice also have decreased expression of PDI [45, 46]. Decreased expression of PDI in response to high glucose may alter protein maturation in the ER, triggering a stress response which includes increased protein degradation by the proteasome. The mechanism of decreased PDI expression in mesangial cells by acute exposure to high glucose remains to be defined.

In conclusion, the proteomics data and bioinformatic data analysis suggests that murine GMCs respond to acute HG via expression of proteins related by pathways regulating protein posttranslational modification and protein stability. These acute differences may also be important for cellular function as reported for GMCs treated with longer more chronic hyperglycemic time points of differences in specific protein abundance such as enolase, actin, and annexin proteins [30].

Conflict of Interests

The authors have no conflict of interests to disclose.

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Review Article

Immune Cells and Inflammation in Diabetic Nephropathy

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Diabetic nephropathy (DN) is a serious complication of diabetes. At its core, DN is a metabolic disorder which can also manifest itself in terms of local inflammation in the kidneys. Such inflammation can then drive the classical markers of fibrosis and structural remodeling. As a result, resolution of immune-mediated inflammation is critical towards achieving a cure for DN. Many immune cells play a part in DN, including key members of both the innate and adaptive immune systems. While these cells were classically understood to primarily function against pathogen insult, it has also become increasingly clear that they also serve a major role as internal sensors of damage. In fact, damage sensing may serve as the impetus for much of the inflammation that occurs in DN, in a vicious positive feedback cycle. Although direct targeting of these proinflammatory cells may be difficult, new approaches that focus on their metabolic profiles may be able to alleviate DN significantly, especially since dysregulation of the local metabolic environment may well be responsible for triggering inflammation to begin with. In this review, the authors consider the metabolic profile of several relevant immune types and discuss their respective roles.

1. Introduction

Diabetic nephropathy (DN) is the most common complication and leading cause of mortality associated with diabetes [1]. DN is a leading contributor to cases of kidney failure in developed countries, and both type I and type II forms of diabetes can result in DN [2]. While the global pattern of DN incidence is not fully surveyed, it is nonetheless a widespread occurrence presently that is expected to increase in prevalence [3]. Numerous factors, both environmental and genetic, have been reported to influence DN onset, severity, and the rate of progression. DN is assessed clinically through a five-stage system of criteria, with each stage featuring a distinct set of functional and structural changes and reflected alterations in benchmark renal function markers [4]. The structural changes begin with glomerular and tubular hypertrophy and the thickening of the basement membrane and mesangium expansion, leading to end-stage glomerular closure and tubulointerstitial fibrosis [5, 6]. These changes are driven primarily by the dysregulation of typical glucose metabolism pathways, leading to the characteristic loss of blood glucose control and aberrant adipose function [7–9]. This loss of glucose control can lead to a number of other changes, including inflammation and cellular stress.

While perhaps not as dramatically observed as in some other conditions, inflammatory processes are heavily involved in the structural deterioration that occurs in DN. This involvement is only natural given that inflammation is known to be involved in the pathogenesis of diabetes, with elevation of serum inflammation markers in long term diabetes patients [10]. The cause(s) of inflammation in DN are not clear, but some combination of pathogen insult and/or tissue damage may be responsible. The former is not likely to be the strongest contributor however, as the kidney is not typically exposed to pathogens and has not been shown to be more vulnerable during DN. Since it is very likely that the inflammation occurring in DN is sterile and chronic, intrinsic kidney cell injury, namely, injury to glomerular,

tubular, and vascular cells, may be the main cause. Damage or danger signals released by injured renal cells can trigger remodeling processes by stimulating renal cells and activating immune cells of both the innate and adaptive systems. Other metabolic signals may also contribute. In this review, the authors will review the roles played by, and crosstalk between, several T cells, macrophages, dendritic cells, and renal tubular cells, which are among the most important cell types contributing to the inflammation mediated acceleration of DN progression. The authors will consider the impact glucose metabolism and other mechanisms of metabolic control may have on these different cell types as a key explanatory factor for DN pathogenesis.

2. Antigen Presenting Cells

Dendritic cells are the professional APCs in the body, and their function is intimately tied to the progression of diabetic nephropathy. DCs exist primarily in two types: the more common myeloid-derived type (mDC) and the less common, but more highly pathogenic, plasmacytotic (pDC) variety. mDCs and pDCs differ primarily in terms of TNFα production, with the high levels of TNFα secreted by pDCs having been shown to result in significant morphological changes in DN as well as other autoimmune and inflammatory disorders [11]. More importantly however, DCs can act as keen sensors of damage, being armed with a complete repertoire of receptors for both extrinsic pathogens and intrinsic factors. These include most classes of pattern-recognition receptors, including Toll-like receptors, NOD-like receptors, and C-type lectin receptors, which may also coligate [12]. Following activation by these stimuli, DCs can begin initiating an inflammatory response to counter a perceived threat, potentially via NLRP3 or alternative "licensing" [13, 14]. DC linkage with T cells is sufficient to activate the T cell receptor (TCR) and drive adaptive responses. Signaling along these complex pathways generally ends in the activation of transcription factors such as IRF7, NF-κB, and IRAK, which can then coordinate the secretion of varied chemokines to attract PMNs and alter the behavior of other nearby cells [15, 16]. DCs are able to sense extracellularly through cell surface receptors and intracellularly via endosome-surface receptors. These latter receptors, such as TLR8, may also permit DCs to directly sense microRNAs and other molecules taken up as exosomes [17]. Overall, DC phagocytosis and the ensuing processing mechanisms are critical to the initiation of inflammation. DCs are widely present in the renal tubulointerstitium in normal mice, and immunohistochemistry has also revealed the significant numbers of both mDCs and pDCs in normal human kidneys. As such, resident DCs in kidney may be readily capable of processing damaged cells and signals and consequently initiate the cycle of tissue damage and repair present in DN. DCs have been shown to be critically involved in tubulointerstitial inflammation in various progressive nephritic diseases and in the remnant kidney model. Unfortunately however, the role for DCs in DN inflammation has not been well studied, in part due to difficulties associated with plasticity and the relative instability of cultured DC cell lines [18].

Similar to DCs, macrophages also function as APCs, but few resident macrophages are normally present in the kidneys. Curiously however, a heavily increased macrophage presence is observed in both the glomeruli and tubulointerstitium in human T2D, and the prevention of such macrophage infiltration has been the focus of some recent work [19–21]. Such an increase in the macrophage population has been correlated with the degree of glomerulosclerosis, increased proteinuria, increased serum creatinine levels, and the presence of interstitial fibrosis. Importantly, deletion or inhibition of macrophage accumulation by decreasing intracellular adhesion molecule 1, monocyte chemoattractant protein 1, or colony-stimulating factor receptor 1 ameliorated DN in both db/db and STZ-induced diabetic mice [22]. Given that these knockouts primarily affect chemotaxis and late polarization, it stands to reason that the increase in macrophages around the kidney in DN is primarily a result of outside recruitment, and not simply a local population expansion. Similarly, Smad7 deletion, which impacts the TGFβ signaling pathway, has been shown to enhance macrophage recruitment to the kidneys [23]. Since outside recruitment typically only occurs in response to damage sensing, it can thus be expected that these macrophages only come into play some time after the initial damage event(s).

Two main types of macrophages, the classically activated and proinflammatory M1 and the alternatively activated and anti-inflammatory M2, are commonly recognized, with the M2 type being further divided into three subtypes. Macrophage populations are generally highly plastic however, such that rigid/exclusive production and expression patterns are rarely observed [24]. Several authors have also suggested various other unique macrophage populations, based on factors such as the differential expression of Lyc6 [25]. Tissue-resident macrophages may also generally express other markers that are tissue-specific, unlike classical bone-marrow derived macrophages [26–28]. Nonetheless, the simple classification scheme is useful for understanding many basic aspects of macrophage function. Transitions along the spectrum from the two extremes have been an area of particular focus and are also highly relevant to the progression of DN. Many of the infiltrating macrophages in the middle stages of DN are likely to adopt the M1 type and cause significant damage that accelerates the condition, although the precise origin of these macrophages is not fully clear.

Many inducers of M1 activity and M1 signature products have been implicated with DN, with TNFα being perhaps the most notable. TNFα signaling induces production of NF-κB along a JAK/STAT pathway, leading to inflammation. The cytosolic adaptor protein Myd88 conducts the classical pathway for M1 function downstream of TLR signaling. Myd88 may activate various members of the TRAF family, leading to further activation of IRAK as well as IRF7. M1 may also be stimulated via induction of other pattern-recognizing receptors such as NLRs. Regardless of the precise channel used, M1 can be stimulated by both classical pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), and damage-associated molecular patterns (DAMPs) such as extracellular DNA [29]. These stimuli may also elicit different responses in chronic inflammation [30].

The relevance of PAMP-driven signaling to DN mainly lies in possible changes in response to secondary infection, a situation that is not wholly clear. Given the known damage and ECM changes that occur in DN however, it seems that DAMPs are equally important to consider, if not more so.

The transcription factor IRF5 is intimately involved in M1 processes, such that IRF5 is also considered to be one of the defining characteristics of the M1 phenotype [31]. IRF5 is also critical for M1 production of type one interferons and also influences the activity of iNOS, a primary means by which M1 can generate ROS [32]. These signaling molecules work in concert to prevent wound healing but may also prevent hyperresponse via inhibition of other proinflammatory cell types. After all, ROS production has been shown to lead to the inhibition of several T effector subtypes, among others [33]. Despite being proinflammatory, M1 are also sensitive to oxidative stress. M1 are suppressed by an overabundance of NO via the nitration and subsequent loss of function of IRF5 and are actually more abundant in iNOS deficient mice as a consequence [34]. Similarly, hypoxia has been shown to inhibit M1 polarization via altered expression of HIF-1α [35]. Oxidative stress has been shown to lead to changes in autophagy and other macrophage activities as well. Interestingly however, high levels of oxidative stress observed in DN have been previously shown to result in tubular cell and podocyte injury, and the debris from cells killed in such manner may also serve to further stimulate M1 and overcome the inhibitory pressure M1 may experience otherwise.

M1 may also produce sizeable amounts of IL-6, but the precise importance of this production is unclear given the fact that many other cell types also secrete the cytokine during inflammation. M1 macrophages can also form inflammasomes to further their function, with the NLRP3 and AIM2 varieties being the ones best characterized thus far. These inflammasomes may form under a wide variety of stimuli and gather together large amounts of caspase 1, which can then cleave and activate interleukins 1beta, 18, and 33 [36]. CARD and PYR domains on these inflammasomes may recruit and involve a broad assortment of different proteins to further M1 function. Damage sensing by M1 macrophages has been demonstrated to contribute to such inflammasome activation, although the exact molecular machinery may be varied and has not been completely characterized [37]. The precise duration of inflammasome activation is also not well understood.

Metabolic control of M1 has become subject of increasing interest and may be particularly relevant to DN given the metabolic disruption that is implicit in the condition. Recent work has shown that M1 macrophages may have an altered expression of the Krebs cycle enzyme isocitrate dehydrogenase, making it a possible target [38]. This altered expression may have important ramifications given the change in glucose availability present in DN. The glucose and salt sensor aldose reductase has also been shown to play a key role in M1 metabolic control, although it is not fully clear if its control is mostly mediated by its production of sorbitol or through other protein interactions [39]. High salt has been demonstrated to result in increased inflammasome activity, but it is not clear if the response is to the salt itself or just to osmolality increase [40]. Advanced glycation end products, commonly observed in diabetes, can also strongly influence M1 macrophage function [41]. It is clear, however, that the provision of high amounts of glucose is a facilitator for M1 stimulation, partly mediated through the increased expression of SREBP1 [42]. These changes may in part be mediated by mitochondrial changes [43]. Interestingly, additional stimulation using insulin may significantly reverse these additions, such that they remain responsive along the standard pathways of metabolism. Arginine metabolism has also been suggested to be involved in M1 control, while other metabolites are probably also relevant to uncertain capacities [44].

In addition, as part of their phagocytotic capacity, macrophages may also uptake large amounts of cholesterol to become foam cells. Foam cells have been identified to occur in a number of renal conditions, and some recent work has focused on promoting cholesterol efflux to prevent foam cell buildup, but it remains to be seen if foam cells should truly be targeted or simply regarded as a marker [45]. In addition, the nuclear receptors FXR, LXR, and PXR have been identified to have important function in regulation of M1, particularly in the Kupffer cells of the liver and in resident macrophage populations in the kidneys. Activation of the bile acid receptor FXR has been shown to repress macrophage function and infiltration capability in DN, partly by suppressing the IFNγ mediated activation of STAT1 [46]. However, FXR activation may not be wholly anti-inflammatory, with its mechanistic control still not fully understood. LXR function in macrophages has largely been explored in the context of atherosclerosis, in which LXR activation has been reported to induce efflux of cholesterol from macrophages [47]. LXR activation also influences the activity of the transcription factors IRF8 and Pu.1 [48]. These effects have been confirmed in DN, with LXR activation leading to the amelioration of the condition [49]. This amelioration might be highly dependent on macrophage function, as the AP-1 and NF-κB signaling pathways have been shown to be important in DN, since those pathways feature prominently in macrophages. PXR may also influence macrophage sensitivity to cholesterol, although it may play a more important role in regulating the metabolism of the drugs being used to treat DN given its general role in controlling drug responses [50]. At the same time, activation of these nuclear receptors may also lead to the induction of PPAR family members such as PPARα, which can then serve to negatively regulate inflammation [51]. PPARα has also been shown to influence the self-renewal of erythrocyte progenitors [52]. The balancing of the proinflammatory effects metabolites may have and the anti-inflammatory effects of their activated receptors remains to be clarified.

Overall, macrophages tend to trend towards M1 at the onset of inflammation and then switch to M2 to promote healing some time thereafter. The precise mechanisms that induce such a switch are not understood, although damage sensing, further cytokine stimulation, and autocrine regulation have been proposed as possible reasons [53–55]. Changes in the expression of surface markers such as from CD11b to CD163 may be used to monitor these changes in part but are not wholly sufficient given the persistent expression of perceived M1 markers well after a change in cytokine

production has been observed. A large and varied assortment of compounds have attempted to target this transdifferentiation process as a means for alleviating the inflammation in DN and other autoimmune disorders, but none have been extremely successful thus far, despite revealing some interesting target options [56]. Many of these compounds have focused on repressing the activity of M1 transcription factors through selective inhibition, but it is not clear if the transition is actually controlled along a specific pathway. The precise order in which these changes occur is also not fully known.

M2 macrophage differentiation has been one of the key goals for immunology-based treatments for DN, due to the protective effects M2 may induce. In particular, M2 are responsible for the secretion of IL-10, a potent cytokine that can act to suppress the activity of most proinflammatory cell types. IL-10 function runs counter to that of TNFα, helping to shut down its efficacy, partly by disrupting the p38/MAPK pathway [57]. IL-10 may also lead to the suppression of iNOS activity via the induction of HO-1 and consequent CO salvage [58]. In addition, IL-10 mediated induction of a PI3K/AKT pathway for cell survival may directly improve wound healing despite otherwise hindering proliferation [59]. Polyamine secretion from M2 may also encourage the resolution of inflammation by influencing transcription programs in other cells [60]. Curiously, M2 function is coordinated to a large extent by IRF4, which is typically understood to be a proliferative and proinflammatory transcription factor across a wide range of different cell types. The exact signal transduction network in play within M2 macrophages that reverses that typical functional pathway is unknown.

3. T Helper Cells

T helper cells become relevant at the site of inflammation later than APCs but still have an important functional role in regulating inflammation resolution or lack thereof. T helper cells do not typically have as strong of a residency status as other immune cells, but some tissue-specific markers have been identified. Once activated, CD4+ T cells can organize many of the hallmark signs of inflammation, such as widespread recruitment and chemokine release [61]. Of particular interest in DN is the fact that CD4+ T helpers have been shown to engage in significant crosstalk with fibroblasts to influence fibrosis [62]. As such, targeting against several of these subsets has been shown to be useful in treating DN, particularly Th17 and Th1 [63]. T helper cells can be conventionally split into T effector or T regulatory phenotypes, with the proinflammatory Th1, Th2, Th17, Th9, and Th22 subsets forming the CD4+ subset of the former. Each of these subtypes has the capability of producing signature cytokines, although such production is not necessarily exclusive. Lineage-defining transcription factors may be highly expressed and critically regulate the function of these subtypes, with dynamic interplay between them potentially contributing to the nature of their polarization. The precise strength of TCR signaling at the beginning of their activation has been shown to bias future differentiation towards certain phenotypes [64, 65]. Different metabolites have been demonstrated to also have variable impact across these different phenotypes, although many of those differences remain undiscovered. CD4+ T cells can also be highly plastic, adopting multiple phenotypes either over time or even simultaneously [66]. As such, it is useful to first consider all of the CD4+ cells in context before examining the particular factors that a particular phenotype may hold somewhat exclusively.

CD4+ T cell activation occurs along a multistep pathway, with bona fide activation being somewhat ambiguous. The energy need during this differentiation process, while not well studied, is likely to be quite high, given the clear disparity in metabolic intake between differentiated T effector types and naïve Th0 cells. During this differentiation process, cells may be directly activated and subsequently polarized or potentially undergo a "failed" activation and instead enter into a state of anergy. The nature of CD4+ anergy is not well known, with much of the current information on this phenomenon having been gleaned from analogous study on CD8+ T cells. Anergetic T cells are understood to have very weak, if any, capability of sending further stimulation to other cells, especially via cytokine production [67]. Several types of viral infections are known to induce T cell anergy as a means of escaping immune suppression, and TGFβ signaling is thought to be involved, but much remains to be clarified. Intriguingly, TLR7 activation following damage sensing has been suggested to induce this state of anergy, implying a potential increase in the population of anergetic T cells in the context of DN [68].

Following activation, T helpers can then pass through a functional stage of variable duration before eventually making a choice to become memory cells or otherwise undergo exhaustion or apoptosis. CD4+ T helper memory is thought to be critically regulated by TSLP, which may induce the cells to adopt a lower metabolic profile [69]. More recently, a newer exhaustion phenomenon has been uncovered, which has been treated as being distinct from simple plastic transitions across different phenotypes [70]. CD4+ T cells are known to require significant autocrine secretion of IL-2 (as well as lesser amounts of IL-5 and IL-7) in order to maintain their viability and continue functioning [71]. In some viral diseases however, T cell function has been observed to fade over time, with IL-2 autocrine signaling being suppressed [72, 73]. This suppression has been correlated with the increased expression of the cell surface marker PD-1 and the transcription factor Bcl6, among others [74]. While some early speculation held that these cells were in fact merely becoming Tfh-like, the observation of other markers such as Tim-3 suggests otherwise [75]. Once CD4+ cells begin to undergo exhaustion, they gradually lose functions one at a time. Recently, several authors have proposed that this type of exhaustion may also occur in cases of chronic inflammation, where the continued activation and function of CD4+ cells may eventually be suppressed through some sort of internal transition. This type of exhaustion-mediated transition has thus far been identified to occur between Th17 and Treg phenotypes but may also occur between other T effector types and T regulatory types [70]. This type of exhaustion is likely caused by some kind of

damage sensing mechanism or perhaps the extreme buildup of stress that may occur in chronic inflammation. Deprivation of sufficient nutrients/metabolites may also contribute to exhaustion occurring.

CD4+ T helper tolerance of glucose has been shown to be an important factor in the pathogenesis of diabetes and as such is also highly relevant to DN. In particular, Th17 and Th1 cells have been identified as contributing factors. Th17 cells are a highly active T cell population classically activated through the cytokines IL-6 and TGF-β or alternatively activated into a pathogenic form via IL-1β, IL-6, and IL-23. As noted previously, these cytokines are often present in significant concentrations in the local environment during inflammation, with significant amounts of them being released by epithelial cells, particularly by those under stress. Once Th17 cells receive signaling along either tract, a JAK/STAT pathway is activated, culminating in STAT3 activation of RORγt and ensuing production of IL-17 [76]. Changes in the production of IL-17 and in the expression of RORγt are understood to be the most conclusive indicators of change in Th17 function, but it is also possible that other changes may emerge even earlier. Th17 function has been explored as a possible contributor to worsening DN, but the actual strength of the connection is unclear. Some studies have suggested that Th17 cells might be uniquely upregulated in DN, although it is also possible that the increase is simply a part of a general increase in proinflammatory cytokines [63]. Th1 cells are a similar proinflammatory subset that often responds in a similar fashion to Th17 cells in the face of stimulus. By contrast, Th1 cells produce large amounts of IFNγ and express the transcription factor T-bet (Tbx21). Stimulated by the common APC product IL-12p40, Th1 cells rely on STAT4 activation at the end of a JAK/STAT pathway to function. Like other T helper subtypes, Th1 are also influenced by BATF-driven and IRF4-driven transcription networks [77]. Th1 cells have also been shown to be likely candidates for upregulation in DN.

CD4+ T helpers may also respond differently to common forms of oxidative stress common in DN. Th17 cells may also be preferentially activated by the hypoxia environment that can form in DN, although they are also susceptible to inhibition by nitric oxide and other ROS stress [78]. In this manner, they differ from other CD4+ T helpers which are less responsive to NO or otherwise feed off of it to increase inflammation, such as Th9 [79]. Unlike APCs, T helper cells have significantly muted expression of classical DAMP and PAMP receptors, such that they are not as likely to be directly influenced by danger signals such as extracellular DNA. Recent work has shown that human T helpers may be inhibited via activation of TLR7 however, raising hope that other direct influences may also be uncovered. APC transduction of such signaling might occur at a rapid pace in DN however, especially given the broad scale damage that is present during accelerated remodeling. In addition, T cells can directly receive signaling via other extracellular metabolites, such as ATP [80]. Extracellular ATP may then activate the AMPK pathway to increase inflammation by upregulating metabolism [81]. While it is not intuitively obvious that increases in metabolism must correlate with an increase in inflammation, it is almost always the case, as basic increases of metabolism can strongly impact cellular activation [82].

Metabolic control of CD4+ T helpers has been the focus of increased study in recent years, with many metabolic products having been identified to bias cellular polarization. For instance, succinate, an intermediate in the Krebs cycle, has been shown to play an important role in modulating Th17 activity [83]. Provision of excess amino acids has also been shown to somewhat preferentially induce Th17 activation, while its opposite starvation selectively inhibits the phenotype [84, 85]. Other metabolites, such as salt, have also been shown to induce T effector activity, although it is also possible that the change is primarily a result of an increase in osmolality [86, 87]. Glucose can similarly lead to the creation of phenotype bias [88]. Interestingly, in many of these cases, the Th2 and Treg phenotypes are not affected, for reasons not fully clear. One particularly striking example of this phenomenon is the fact that BRD4 inhibition seems to sharply limit Th1 and Th17 activity while leaving Th2 and Treg populations at original levels, despite the fact that BRD4 is broadly expressed across CD4+ lineages and is a very general factor critical for the assembly of the superelongation complex for replication and transcription [89, 90]. It may also be possible that CD4+ T helpers can respond differently to the provision of nucleotides or other metabolites of different classes. Regardless, this differing need for various metabolites leads to a useful potential source for T helper targeting or at the very least cautions against the use of some methods for treatment. For instance, the inhibition of Th17 cells has been a longstanding goal in many conditions besides DN, and one of the prime means for achieving this has been through the treatment of broad-acting corticosteroids. Unfortunately however, recent research suggests that Th17 cells may actually be able to more effectively respond to corticosteroid stress than some other subsets and actually survive such inhibition over the short term [91]. As such, it is important to observe that these metabolic differences can only be targeted in cases where one cell type expresses and requires a metabolite that other polarization states do not also need.

4. Tubular Cells

While not immune cells, tubular cells in the kidney may serve to propagate many of the immune signals present in DN, both through direct cytokines and through metabolites. After all, tubular cells handle filtered glucose and other metabolites, while also transporting electrolytes and water. Although currently there are debates about whether or not the primary role of tubular cells is acting in parallel with glomerular cells in the pathogenesis of DN, it is a well received fact that functional dysregulation in the tubules and cell injury are main contributors to DN [92]. As noted previously, one of the early pathologic changes in DN is tubular cell hypertrophy, followed by increased thickness of tubular basement membranes. These changes affect the composition of the extracellular matrix and drive feedback for further changes [93]. Subsequently, tubular atrophy, atubular glomeruli, extensive immune cell infiltration, and fibrosis develop and progress

[94]. Multiple factors including hypermetabolism, excessive free radicals, and chronic inflammation have been implicated in DN tubulointerstitial lesions. Naturally, work load in tubular cells in diabetes is greatly elevated as a result of hyperfiltration, along with accumulated high glucose and related metabolites. Accordingly, energy generation and consumption are increased to meet the functional need, leading to a state of hypermetabolism that provokes cell stress and free radical overgeneration [95]. Moreover, nonenzymatic interactions between high glucose pressure and proteins promote the generation of carbonyls in the process, as well as advanced glycation end products which provoke free radical synthesis [96]. This cycle may also cause positive feedback in the absence of a functional response to insulin [97]. Tubular cells may also directly produce TGFβ in response to glucose to directly modulate immune function [98].

Free radicals are widely recognized to be critical for DN pathology. Free radicals may directly damage cell structures and modify cellular proteins and DNA, resulting in cell stress and injury, which may subsequently activate DCs and macrophages to initiate a pattern of damage associated sterile inflammation [10]. Additionally, free radicals may oxidize nitric oxide (NO) to decrease NO availability and cause vasoconstriction, leading to decreased tubular blood supply and hypoxia. Hypoxia suppresses prolyl hydroxylase domain proteins and activates oxygen sensing transcription factor hypoxia inducible factor (HIF-1α). It is noted that free radicals can also increase HIF-1α transcription and function by directly activating the HIF-1α promoter and regulating hydroxylase function of the asparaginyl hydroxylase [99]. Indeed, the levels of HIF have been noted to increase in the tubular and interstitial cells of DN, and the suppression of HIF has been shown to ameliorate DN [100]. One of important functions of HIF is to switch cell metabolism from oxidative phosphorylation to glycolysis to increase cell survival under hypoxic condition. However, this metabolic shift also causes the changes in immune cell phenotypes that favor inflammation. HIF-family proteins increase macrophage aggregation, invasion, and mobility and are intimately involved in macrophage polarization. For instance, macrophages lacking HIF-2α cannot be stimulated via LPS to generate M1 inflammatory responses. The function of HIF in DCs is less clear. Hypoxia induces the production of proinflammatory cytokines TNFα and IL-1β by DCs. On the other hand, DCs cultured under hypoxia have less costimulatory molecules and maturation markers and show decreased migration in response to C-C chemokine receptor type 7 ligands [101].

Since the process of T cell activation and differentiation is tightly associated with metabolic switch, it is not unexpected to find a role for HIF in T cell activation and differentiation. Th1, Th2, and Th17 cells exhibited increased glycolysis and less oxidative phosphorylation compared to T regulatory cells, which show a greater reliance on lipid oxidation and oxidative phosphorylation [102]. Th17 has high levels of HIF-1α and deletion of HIF-1α in T cells inhibits Th17 differentiation in vitro and in experimental autoimmune encephalomyelitis [103]. In contrast, hypoxia and deletion of HIF ubiquitination ligase VHL both promote stabilization of HIF-1α and enhance generation of Th17 CD4+ cells. The generation of Treg is usually opposite to the induction of Th17 CD4+ cells. Thus, enhanced Treg cell differentiation was observed in the case of HIF-1α deletion, a condition not favoring Th17 cells lineage differentiation [104]. One possible molecular mechanism for HIF attenuation of Treg cell development is direct binding and targeting of the Treg lineage-defining transcription factor Foxp3 for degradation [105]. HIF-1α can also affect metabolic adaptations in CD8+ T cells and control expression of many molecules associated with effector function and migration [106]. HIF-1β-deficient T cells show diminished expression of effector molecules. Overall, HIF activation by hypoxic microenvironment as well as free radicals in tubulointerstitial area favors inflammation via modulation of immune cells.

The levels of HIF are also elevated in tubular cells in DN. It is unknown if HIF also directly promotes the inflammation in tubular cells. However, since the preferred metabolic fuels for tubular cells are not glucose, the act of HIF on these cells may differ. Nevertheless, phenotypic alteration of tubular cells to proinflammatory has been observed in DN. This may contribute directly to local inflammation. Both high glucose and advanced glycation end products can activate NF-κB and inflammasome and upregulate MCP-1 and VCAM-1 in tubular cells, while also promoting further ECM modifications [107]. Large amounts of albuminuria have also been implicated in the proinflammatory phenotype of tubular cells [108].

Finally, apart from genetic susceptibility, DN takes about 10–20 years to develop as the result of multiple vicious interplays among dysregulated metabolism, cell stress and injury, chronic inflammation, and epigenetic changes. Although we have gained some knowledge about the pathogenesis of DN and have developed some treatment in recent years, at present we are still unable to prevent disease progression to diabetic renal failure. As such, it is critical to understand the details of metabolic abnormalities, the pros and cons of HIF activation, and the keys to block chronic inflammation in order to enable more effective treatment of DN.

Conflict of Interests

The authors do not have any conflict of interests regarding the publication of this paper.

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Research Article

Renal Protection by Genetic Deletion of the Atypical Chemokine Receptor ACKR2 in Diabetic OVE Mice

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In diabetic nephropathy (DN) proinflammatory chemokines and leukocyte infiltration correlate with tubulointerstitial injury and declining renal function. The atypical chemokine receptor ACKR2 is a chemokine scavenger receptor which binds and sequesters many inflammatory CC chemokines but does not transduce typical G-protein mediated signaling events. ACKR2 is known to regulate diverse inflammatory diseases but its role in DN has not been tested. In this study, we utilized ACKR2$^{-/-}$ mice to test whether ACKR2 elimination alters progression of diabetic kidney disease. Elimination of ACKR2 greatly reduced DN in OVE26 mice, an established DN model. Albuminuria was significantly lower at 2, 4, and 6 months of age. ACKR2 deletion did not affect diabetic blood glucose levels but significantly decreased parameters of renal inflammation including leukocyte infiltration and fibrosis. Activation of pathways that increase inflammatory gene expression was attenuated. Human biopsies stained with ACKR2 antibody revealed increased staining in diabetic kidney, especially in some tubule and interstitial cells. The results demonstrate a significant interaction between diabetes and ACKR2 protein in the kidney. Unexpectedly, ACKR2 deletion reduced renal inflammation in diabetes and the ultimate response was a high degree of protection from diabetic nephropathy.

1. Introduction

Although hyperglycemia is the initiating and essential cause for all diabetic complications there is accumulating evidence that inflammatory processes activated by chronic elevated glucose are integral to the development of diabetic complications [1]. Diabetic nephropathy (DN) is one of the most severe and common complications of diabetes and it is the leading cause of end stage renal failure in the world. Immune modulation and inflammatory process contribute to the development and progression of DN [2, 3]. In diabetic kidneys expression of proinflammatory chemokines rises and infiltration of inflammatory cells increases [4–7]. These changes are correlated with progression of tubulointerstitial injury and deterioration of kidney function [8–10]. Inhibition of renal inflammation by small molecule inhibitors or by antibodies directed against chemokines or chemokine receptors has been shown to reduce renal damage in DN [11–14]. More complete understanding of how the kidney modulates immune and inflammatory processes in diabetes may lead to the discovery of improved biomarkers and new therapeutic targets for treatment of DN.

ACKR2 is a chemokine decoy receptor [15] which can bind and internalize chemokines without activating an intracellular response [16]. ACKR2 binds most inflammatory CC-chemokines (CCL2, CCL5, CCL3, CCL4, CCL7, CCL8, CCL11, CCL13, CCL17, CCL22, CCL23, and CCL24) leading to their degradation, thereby reducing local levels of inflammatory chemokines. This makes ACKR2 a likely modulator of local inflammation. The function of ACKR2 has been tested in knockout animals in which deletion of ACKR2 coding sequences increased the inflammatory response in cutaneous

tissue [17], placenta [18], lung [19], liver [20], and colon [21]. The role of ACKR2 has not been examined for a complication of diabetes. In this study, we examined the effect of crossing an established ACKR2 knockout mouse (designated herein as ACKR2 mice) with the diabetic mouse model, OVE26 (OVE). This diabetic model exhibits several features of human DN [22] and extensive renal inflammation [23, 24].

2. Methods

2.1. Animals. All animal procedures followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee. ACKR2 mice on the C57BL/6 background originally from Charles River Italia (Calco, Italy) [17] were bred to FVB mice for at least 10 generations to transfer the ACKR2 deletion to the FVB background (henceforth designated as ACKR2). These ACKR2 mice were bred for two generations to diabetic OVE mice on the background FVB to produce OVE mice homozygous for the ACKR2 deletion (OVE-ACKR2). Mice were maintained up to 6 months of age. Animals had free access to standard rodent chow and water throughout the study.

2.2. Glucose and Albumin Assays. Glucose was assayed in serum samples obtained from nonfasted mice at 6 months of age by the Glucose (HK) Assay Kit (Sigma-Aldrich). At 2 months urine glucose was evaluated with Clinistix (Bayer). Albumin was measured from spot urine samples with a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX) within the linear range of the assay. Urine creatinine was measured with a creatinine assay kit (DICT-500, BioAssay Systems). Urine albumin was expressed as the ratio of albumin to creatinine (μg/mg).

2.3. Assessment of Renal Fibrosis and Inflammatory Cell Infiltration. Kidneys were fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Sagittal tissue sections from the center of the kidney were stained with Masson's trichrome using standard protocols. Stained slides were imaged with a 20x objective. Fibrosis was semiquantitatively scored by a blinded observer for the number of blue stained fibrotic areas per section. Renal inflammatory cell infiltration was evaluated by staining sections with rat anti-mouse CD45 antibody (Angio-Proteomie, Boston, MA). Positive staining was detected with HRP conjugated second antibody and diaminobenzidine (DAB). CD45 positive cell infiltration was evaluated by quantitating the DAB stained pixel area in 8 random, nonoverlapping 200x image fields from the cortical region per mouse with 3 mice per group. Digital images were taken by an observer blind to the identity of the section and the number of positive pixels was quantified by another observer blind to section identity. Pixel number was determined using the ability of Adobe Photoshop to select areas of matching color intensity.

2.4. Microarray Hybridization and Gene Expression Analysis. RNA extraction was done with the RNeasy Mini Kit (Qiagen, Santa Clarita, CA, USA) from frozen kidneys. Extracted RNA was checked for quality on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). The RNA samples having RNA integrity number (RIN) above 8.8 (average 9.1) were used for probe preparation. A 100 ng aliquot of RNA from each mouse was used for probe preparation with an Ambion WT Expression kit. The kit generates sense-strand cDNA from total RNA for fragmentation and labeling was done with an Affymetrix GeneChip WT Terminal Labeling Kit (PN90067). Probes from 3 six-month-old female mice in each group were hybridized to Affymetrix mouse gene 1.0 ST exon arrays and scanned with a GCS 3000 7G scanner and signals were analyzed with Command Console software (Affymetrix, Santa Clara, CA). Gene expression profiles were uploaded to Ingenuity software (Ingenuity Systems, http://www.ingenuity.com/, Ingenuity Pathway Analysis, Redwood City, CA) for data analysis. Gene array data was uploaded to GEO and the access number is GSE51205.

2.5. Quantitative Reverse Transcription-PCR. Total RNA was extracted from whole kidney using TRIzol reagent (Invitrogen, Carlsbad, CA). The cDNA was synthesized with high-capacity cDNA archive kit (p/n 4322171, Applied Biosystems, Foster City, CA) and PCR was performed on an Applied Biosystems 7300 thermocycler with commercially available Taqman reagents (Assay on Demand, Applied Biosystems) for ccbp2 (ACKR2) (Mm00445551_m1), ccl2 (Mm00441242_m1), ccl5 (Mm01302428_m1), ccr2 (Mm04207877_m1), and ccr5 (Mm01216171_m1). Amplification was performed in duplicate using 40 cycles of denaturation at 95°C for 15 sec and primer annealing/extension at 60°C for 1 min. Expression data were normalized to 18s ribosomal RNA (Hs99999901-sl) or GAPDH RNA measured on the same samples. Relative expression ratio was calculated according to the $2^{-\Delta\Delta CT}$ method.

2.6. ACKR2 Immunohistochemistry Staining in Human Kidney. Immunohistochemistry with anti-human ACKR2 antibody was used for detection of ACKR2 expression in human kidneys: renal tissue biopsies ($n = 9$) from diabetic patients with confirmed diabetic nephropathy and 6 nondiabetic control renal tissue samples (2 donor kidneys, 1 normal portion from renal cancer patient, and 3 renal biopsy specimens with proteinuria, lacking visible tubulointerstitial alterations). The research protocol was approved by our Medical Ethics Committee. Tissue was embedded in paraffin, stained with rat anti-human ACKR2 antibody (R&D SYSTEMS, Inc., Minneapolis, MN), and detected with DAB. ACKR2 staining in each section was scored semiquantitatively in tubular and interstitial regions with the criteria of 0 for none, 1 for rare, 2 for some, 3 for common, and 4 for common plus intense. The scorer had no knowledge of group identification of the slides. ACKR2 expression was presented as the average score of each group. In some samples tissues were double labeled with FITC conjugated *Lotus tetragonolobus* lectin (Vector labs), a marker for tubule epithelial cell brush border [25], and the ACKR2 antibody binding was visualized with Cy3 conjugated anti-rat second antibody.

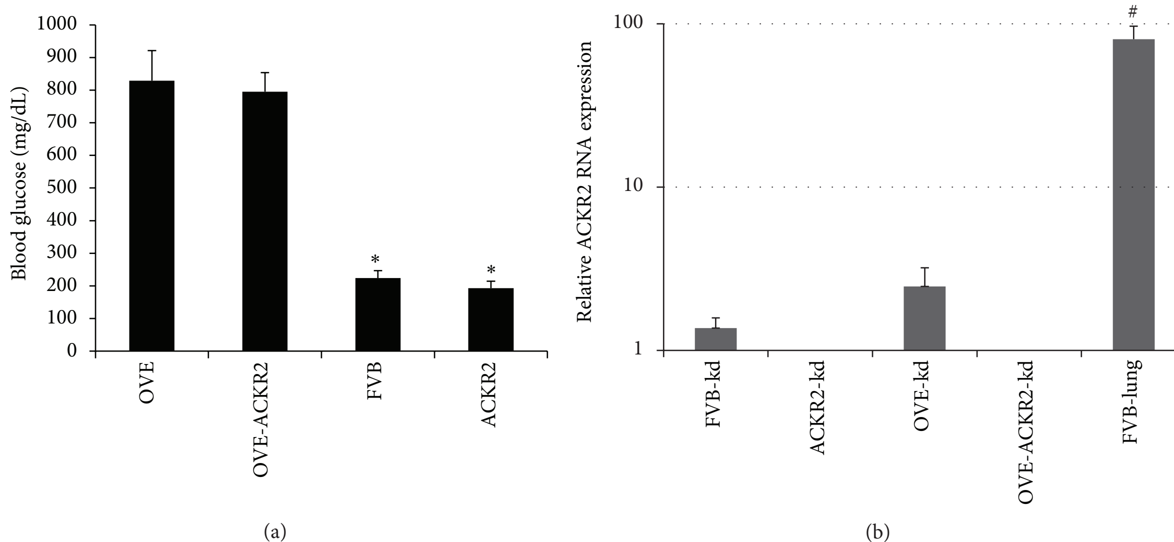

FIGURE 1: Blood glucose and ACKR2 RNA in diabetic and normal mice, with and without deletion of the ACKR2 gene. (a) ACKR2 knockout did not affect blood glucose levels in free fed normal or diabetic mice. $^*p < 0.02$ for both nondiabetic groups versus both diabetic groups. $N = 4, 6, 6$, and 11 in FVB, ACKR2, OVE, and OVE-ACKR2 groups, respectively. (b) Low level ACKR2 RNA expression in kidney is eliminated in ACKR2 KO mice. No ACKR2 RNA was detected in any ACKR2 kidney sample. $N = 4$ for nondiabetic kidney groups, 3 for diabetic kidney groups, and 2 for normal lung. Data are presented on a log 10 graph to include expression values for lung. # indicates that ACKR2 RNA expression in FVB lung was significantly higher than in normal FVB kidney. ACKR2 expression in diabetic kidney tended to be higher than in FVB kidney.

2.7. Statistical Analyses. Data are expressed as means ± SE. Comparisons between two groups were performed by t-test. Comparisons between more than 2 groups were performed by one-way ANOVA. Statistical analyses were performed with SigmaStat software.

3. Results

3.1. ACKR2 Deletion Did Not Alter Diabetes Development in OVE26 Mice. Enzymatic assays, necessary for accurate measurement of blood glucose in OVE diabetic mice [22, 26], indicated that deletion of the ACKR2 gene did not significantly reduce blood glucose levels in 6-month-old OVE-ACKR2$^{-/-}$ mice (Figure 1(a)). Urine glucose, undetectable in normal mice, exceeded 2000 mg/dL at 2 months of age in all OVE and OVE-ACKR2 spot urine samples tested ($n = 4$ per group, data not shown). Expression of ACKR2 RNA was 80% higher in diabetic kidneys compared to normal kidneys (Figure 1(b)), though this difference was not significant ($p = 0.11$). Interestingly high levels of ACKR2 in lungs were observed. Knockout mice served as negative controls for expression analysis.

3.1.1. Knockout of the ACKR2 Gene Reduced Diabetic Albuminuria. Albuminuria was assessed by measuring albumin/creatinine ratio (ACR expressed as μg/mg) in all groups at 2, 4, and 6 months of age (Figure 2). By 2 months ACR was already significantly elevated in OVE mice compared to FVB controls. ACR increased in OVE mice with age, from 600 at 2 months to over 10,000 at 4 months and over 35,000 at 6 months. These values were significantly higher than FVB mice at all ages and significantly higher than ACKR2 mice at 4 and 6 months. Interestingly, ACR values of OVE-ACKR2 mice were significantly lower than OVE values at all ages. The difference between ACR levels of OVE and OVE-ACKR2 groups increased from about 2-fold at 2 months to about 15-fold at 4 months and 7-fold at 6 months.

3.1.2. Reduced Renal Fibrosis and Inflammation in ACKR2 Mice. We evaluated the glomerular and tubular damage in OVE and OVE-ACKR2 mice at the age of 6 months as previously described [22–24]. Trichrome staining (Figure 3(a)) showed that fibrosis in OVE kidneys was much greater than in nondiabetic or OVE-ACKR2$^{-/-}$ kidneys. Semiquantitative scoring of trichrome staining (Figure 3(b)) by an observer blind to genotype confirmed that deletion of the ACKR2 gene significantly reduced fibrosis in diabetic OVE-ACKR2 mice compared to OVE mice.

Infiltration of leukocytes in kidney was determined by staining with anti-CD45 antibody (Figure 4). In nondiabetic FVB and ACKR2 mice, CD45 positive cells were sparsely distributed in the interstitial vessels and in the glomerular tuft. In OVE kidneys many more CD45 positive cells were observed, located mostly in the peritubular, interstitial space in a clustered distribution. Positive staining of CD45 cells was much less evident in kidneys of OVE-ACKR2 mice and appeared similar to staining in nondiabetic mice. Quantitation of CD45 positive pixel area confirmed significantly less leukocyte accumulation in the OVE-ACKR2 mice compared to the OVE mice (Figure 4(b)). Staining for CD3 to identify T cells demonstrated that CD3 positive cells were also more abundant in OVE kidneys than in any other genotype (data not shown).

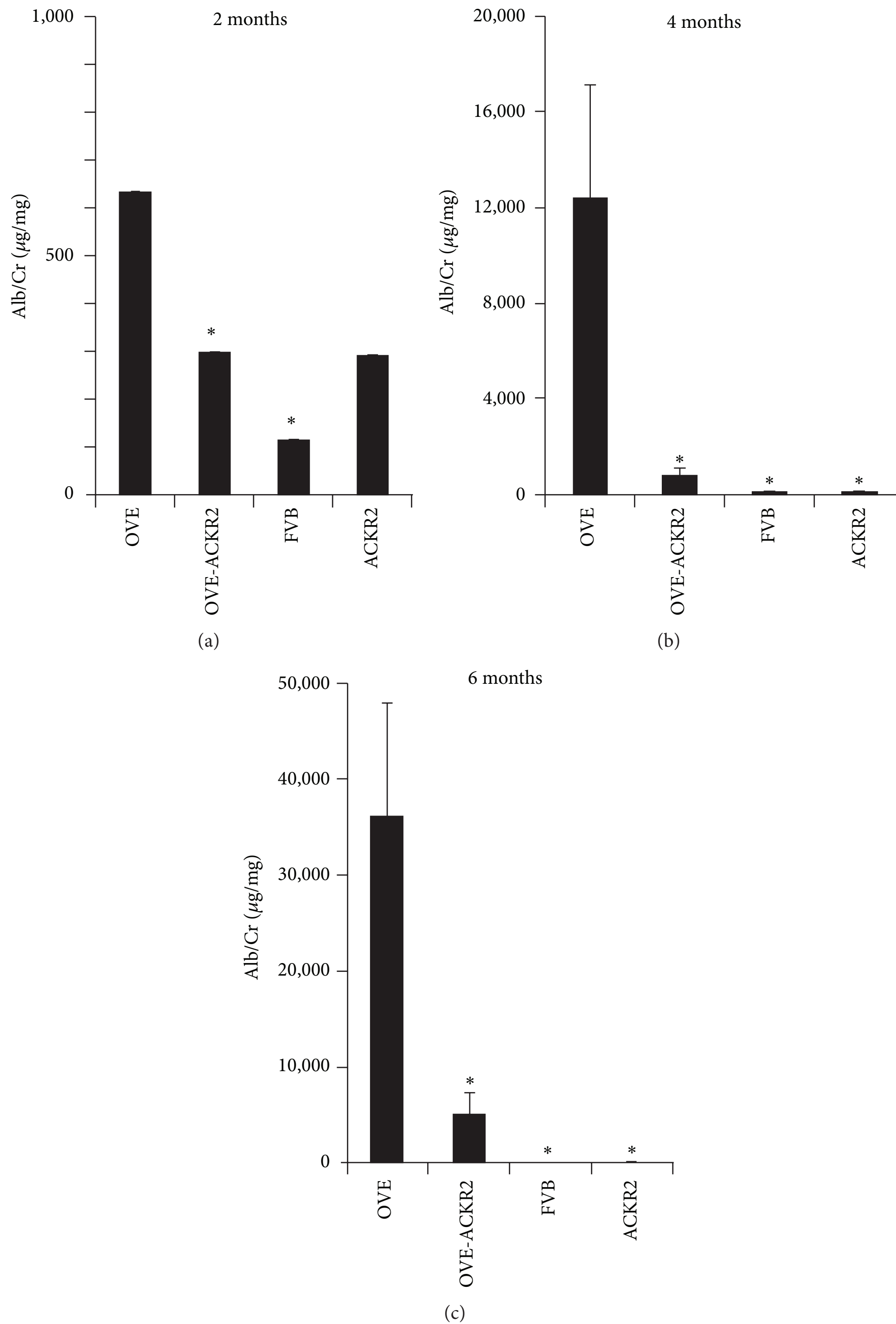

Figure 2: Diabetic albuminuria was reduced by knockout of the ACKR2 gene at 2, 4, and 6 months of age. Urine albumin and creatinine were determined as described in Methods. $*$ indicates $p < 0.05$ versus OVE. Comparisons were performed by one-way ANOVA. $n \geq 12$ in each OVE and OVE-ACKR2 group. For FVB $n = 14, 9$, and 6 at 2, 4, and 6 months, respectively. For ACKR2 $n = 3, 7$, and 7 at 2, 4, and 6 months, respectively.

Inflammatory chemokines CCL2 and CCL5 (ligands for ACKR2) are elevated in DN [6, 27, 28]. Quantitative RT-PCR for CCL2, CCL5, and their receptors was performed on RNA samples extracted from kidneys of all groups at 6 months of age (Figure 5). Levels of CCL2 and CCL5 mRNA significantly increased in OVE mice compared to FVB mice and OVE-ACKR2$^{-/-}$ mice (Figure 5).

3.2. Microarray Analysis of Kidneys from OVE and OVE-ACKR2 Mice. The global changes in gene expression profiles

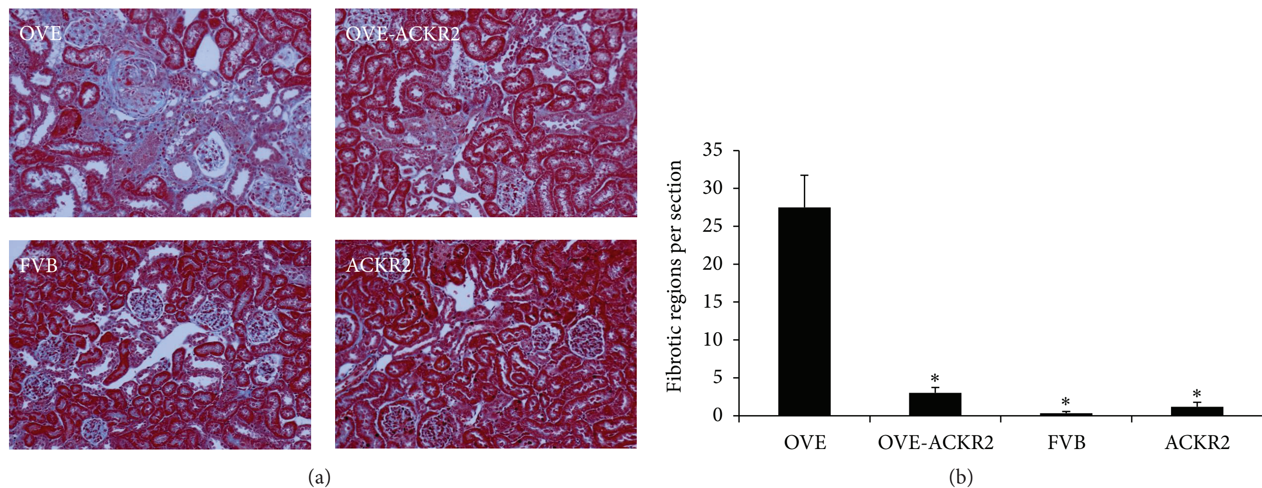

Figure 3: Renal fibrosis is reduced by knockout of the ACKR2 gene in diabetic OVE-ACKR2 mice. (a) Representative images of renal fibrosis illustrated by trichrome staining in a kidney section for each genotype. Original magnification 200x. (b) Scoring of renal fibrosis by blind counting of blue stained fibrotic regions in trichrome stained kidney sections. $^{*}p < 0.02$ versus OVE by one-way ANOVA. 6 sections from 3 mice per group were counted.

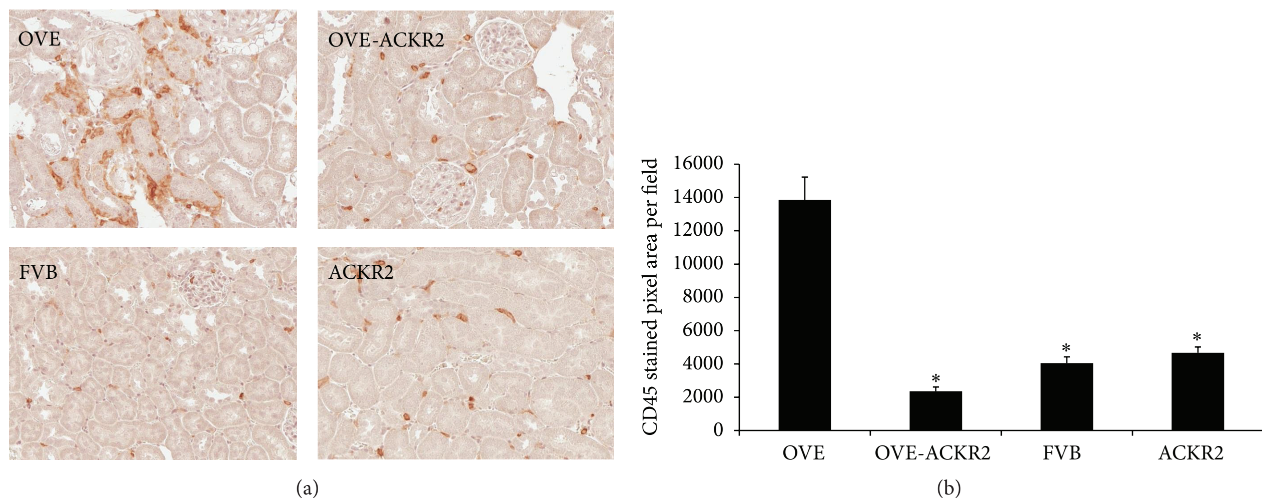

Figure 4: Knockout of the ACKR2 gene reduces leukocyte infiltration in diabetic mice. (a) Representative images of CD45 staining, original magnification 200x. (b) Quantitative analysis of leukocyte infiltration scored as CD45 positive pixel area per visual field. Twenty-four random fields from 3 mice per group were measured. $*$ indicates $p < 0.05$ versus OVE. Statistical comparisons were performed by one-way ANOVA.

were evaluated by microarray. To confirm the reliability of the microarray results correlation coefficients were calculated between RT-PCR and microarray results for CCL2, CCL5, CCR2, and CCR5 based on the 12 samples used in both assays. For all but CCR5 the correlation was at least 0.96 ($p \leq 0.000001$) and for CCR5 the correlation coefficient was 0.6 ($p \leq 0.05$).

Only 18 of 30,000 genes differed at the 0.05 level between the nondiabetic groups, FVB and ACKR2. Therefore, RNA expression of the OVE and OVE-ACKR2$^{-/-}$ diabetic groups was compared to one nondiabetic group, FVB. Using a minimal criterion of 1.5-fold change in expression and a p value of 0.05 versus FVB, there were 715 genes in OVE, 181 in OVE-ACKR2, and 18 in ACKR2 samples that reached this criterion. Expression data was analyzed with Ingenuity Pathway Analysis (IPA) software. Table 1 shows 40 IPA canonical pathways significantly affected by OVE diabetes arranged in 8 biological categories. Signaling pathways for hepatic fibrosis and leukocyte extravasation contained a large number of genes (26 and 27 genes, resp.) altered in expression in OVE samples. This is consistent with the extensive fibrosis and CD45 positive cell infiltration of OVE kidneys (Figures 3 and 4). OVE-ACKR2 kidneys, which showed minimal histological changes, had only 6 induced genes in the fibrosis pathway and 2 in the leukocyte extravasation pathway.

In OVE kidney, many protective pathways such as immune response and cytokine signaling were activated, as indicated by the high number of RNAs with significantly

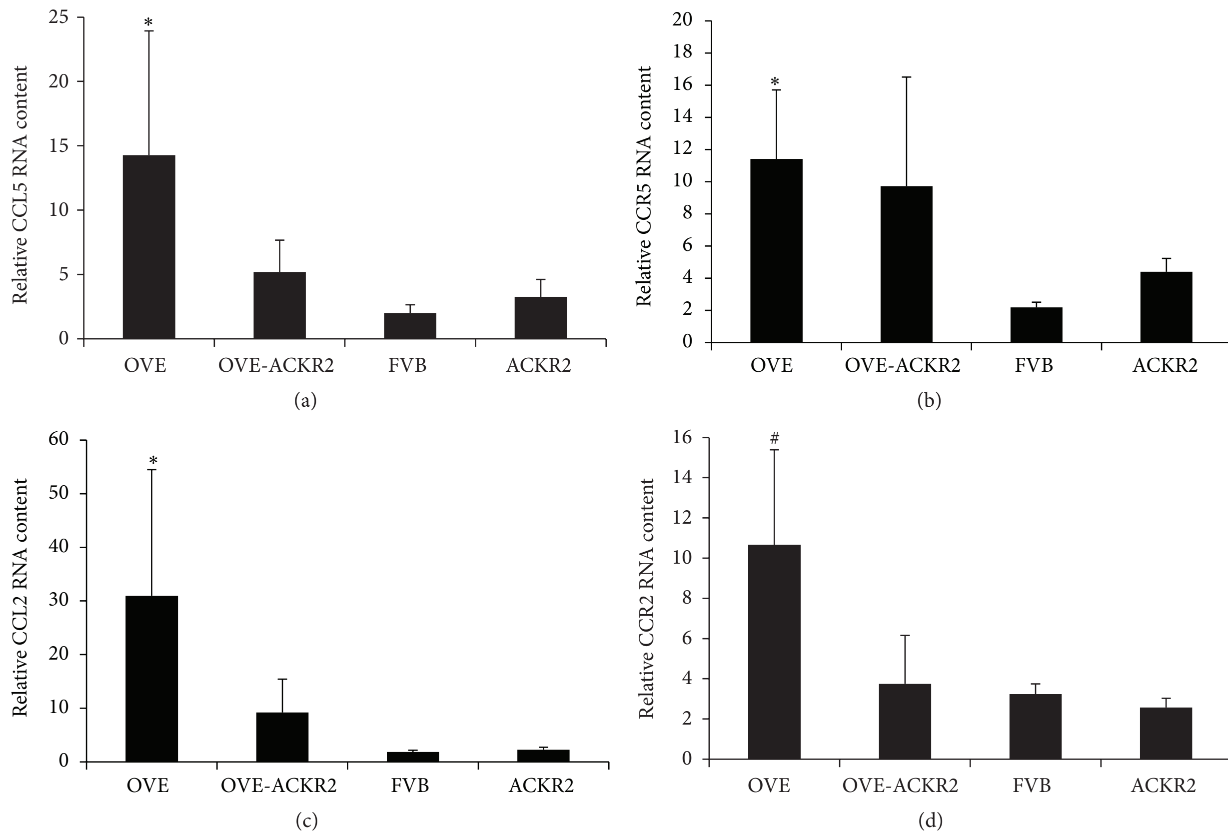

Figure 5: Kidney RNA levels of ACKR2 ligands CCL2 and CCL5 and their receptors. Values were determined by RT-PCR with Taqman probes using 18S as standard. Columns are mean + SE. $n = 3$ OVE, 4 OVE-ACKR2, 5 ACKR2, and 6 FVB. $*$ indicates $p \leq 0.05$ versus FVB and # indicates a trend of $p \leq 0.08$ versus FVB. All determined by one-way ANOVA.

altered expression. The same pathways in OVE-ACKR2 contained only a few RNAs with altered expression. With few exceptions, most of the biological pathways in Table 1 contained at least 4 times as many significantly modified RNAs for OVE as they did for OVE-ACKR2. Also, only 5 of the 40 pathways significantly affected by OVE diabetes were significantly affected by OVE-ACKR2 diabetes. The conclusion that inflammation was reduced by deletion of ACKR2 was also evident at the individual RNA level: transcripts reduced in OVE-ACKR2 kidneys relative to OVE kidneys included RNAs indicative of complement activation (C7 and C1qc) and macrophage and T cell infiltration (Mpeg1, Cd68, and Itgam) and other cytokines (CCL8, CCL9, and CCL28) (Supplementary Tables 1 and 2, in Supplementary Material available online at http://dx.doi.org/10.1155/2016/5362506, show the 50 transcripts most reduced and increased in OVE-ACKR2 relative to OVE, resp.).

3.3. ACKR2 Protein Expression in Kidneys of Diabetic Patients. The effect of diabetes on kidney ACKR2 protein expression was evaluated in human DN and nondiabetic samples using a rat anti-human ACKR2 monoclonal antibody, previously evaluated on human samples [18, 29, 30]. A reliable antibody to mouse ACKR2 is not available. Positive but sporadic ACKR2 staining was visible in diabetic kidneys (Figures 6(a) and 6(d)–6(f)) in tubule epithelial cells and in the interstitium. Stained tubule epithelial cells were positively identified by the presence of a brush border by staining with *Lotus tetragonolobus* lectin [25]. ACKR2 staining was never seen in glomeruli. Positive cells in the interstitium appeared to be either mononuclear cells (lymphocytes or monocytes) or endothelial cells belonging to capillaries or lymphatics. Staining was more frequent and more intense in diabetic samples, which was confirmed by semiquantitative scoring of epithelial cells and interstitial cells (Figure 6(g)).

4. Discussion

This study demonstrates that the ACKR2 chemokine scavenger receptor has an unexpected important role in the development of diabetic kidney disease. Deletion of the ACKR2 gene in OVE diabetic mice produced a great reduction in albuminuria, accompanied by reduced severity of renal fibrosis, leucocyte infiltration, and inflammatory chemokine gene expression. In addition, ACKR2 protein content was elevated in several cell types in kidneys of DN patients.

Chemokines and cytokines regulate the inflammatory processes and contribute to progressive kidney damage in diabetes [31]. Chemokine scavenging has been proposed as a significant mechanism for controlling ongoing inflammation. This suggests that scavenger receptors like ACKR2 could limit DN progression by reducing kidney chemokine levels.

Table 1: Ingenuity pathways in kidney affected by OVE diabetes and/or OVE-ACKR2 diabetes.

Ingenuity canonical pathway	OVE versus FVB		OVE-ACKR2 versus FVB	
	p value	Ratio*	p value	Ratio*
Diseases-specific pathways				
Hepatic fibrosis	$4.27E-10$	26/147	0.003	6/147
Atherosclerosis signaling	$7.76E-10$	23/129	NS	2/129
Altered T cell and B cell signaling in rheumatoid arthritis	$3.38E-08$	17/92	NS	1/92
Graft-versus-host disease signaling	$3.71E-06$	10/50	NS	1/50
Glioma invasiveness signaling	$6.31E-06$	12/60	NS	1/60
Cellular immune response				
Communication between innate and adaptive immune cells	$3.89E-10$	18/109	NS	1/109
Dendritic cell maturation	$2.39E-08$	23/185	NS	0
Altered T cell and B cell signaling in rheumatoid arthritis	$3.38E-08$	17/92	NS	1/92
Pattern recognition receptors of bacteria and viruses	$3.38E-08$	19/106	NS	2/106
Leukocyte extravasation signaling	$6.61E-08$	27/199	NS	2/199
Humoral immune response				
Complement system	$7.94E-11$	13/35	NS	1/35
B cell development	$5.49E-06$	8/36	NS	0
NF-κB signaling	$7.94E-05$	19/175	NS	0
p38 MAPK signaling	0.00017	14/106	NS	0
Antigen presentation pathway	0.0002	7/40	NS	0
Intracellular and second messenger signaling				
p38 MAPK signaling	0.0002	14/106	NS	0
Role of NFAT in regulation of the immune response	0.002	16/198	NS	0
Nitrogen metabolism	0.0037	6/120	NS	1/120
Histidine metabolism	0.0044	7/112	0.00012	5/112
Arginine and proline metabolism	0.0141	8/176	0.00676	4/176
Cellular stress and injury				
Intrinsic prothrombin activation pathway	$1.55E-05$	8/32	NS	1/32
Coagulation system	$1.73E-05$	9/38	NS	0
Extrinsic prothrombin activation pathway	$5.25E-05$	6/20	NS	0
p38 MAPK signaling	0.00017	14/106	NS	0
HMGB1 signaling	0.00245	11/100	NS	0
Cytokine signaling				
Dendritic cell maturation	$2.39E-08$	23/185	NS	0
Acute phase response signaling	$8.91E-08$	25/177	0.00813	6/177
TREM1 signaling	$6.92E-05$	10/66	NS	0
IL-8 signaling	$7.41E-05$	20/193	NS	2/193
NF-κB signaling	$7.94E-05$	19/175	NS	0
Pathogen-influenced signaling				
Dendritic cell maturation	$2.39E-08$	23/185	NS	0
Pattern recognition receptors of bacteria and viruses	$3.38E-08$	19/106	NS	2/106
Virus entry via endocytic pathways	0.00014	13/100	NS	2/100
Clathrin-mediated endocytosis signaling	0.00019	20/195	NS	2/195
Caveolar-mediated endocytosis signaling	0.0015	10/85	NS	1/85
Nuclear receptor signaling				
LXR/RXR activation	$1.63E-13$	28/136	0.0002	7/136
TR/RXR activation	0.0017	11/96	NS	2/96
Aryl hydrocarbon receptor signaling	0.0028	14/159	NS	6/159
Nitrogen metabolism	0.0037	6/120	NS	1/120
LPS/IL-1 mediated inhibition of RXR function	0.0039	18/235	NS	9/235

*Ratio: RNAs altered versus FVB divided by the number of genes in the pathway. NS, not significant.

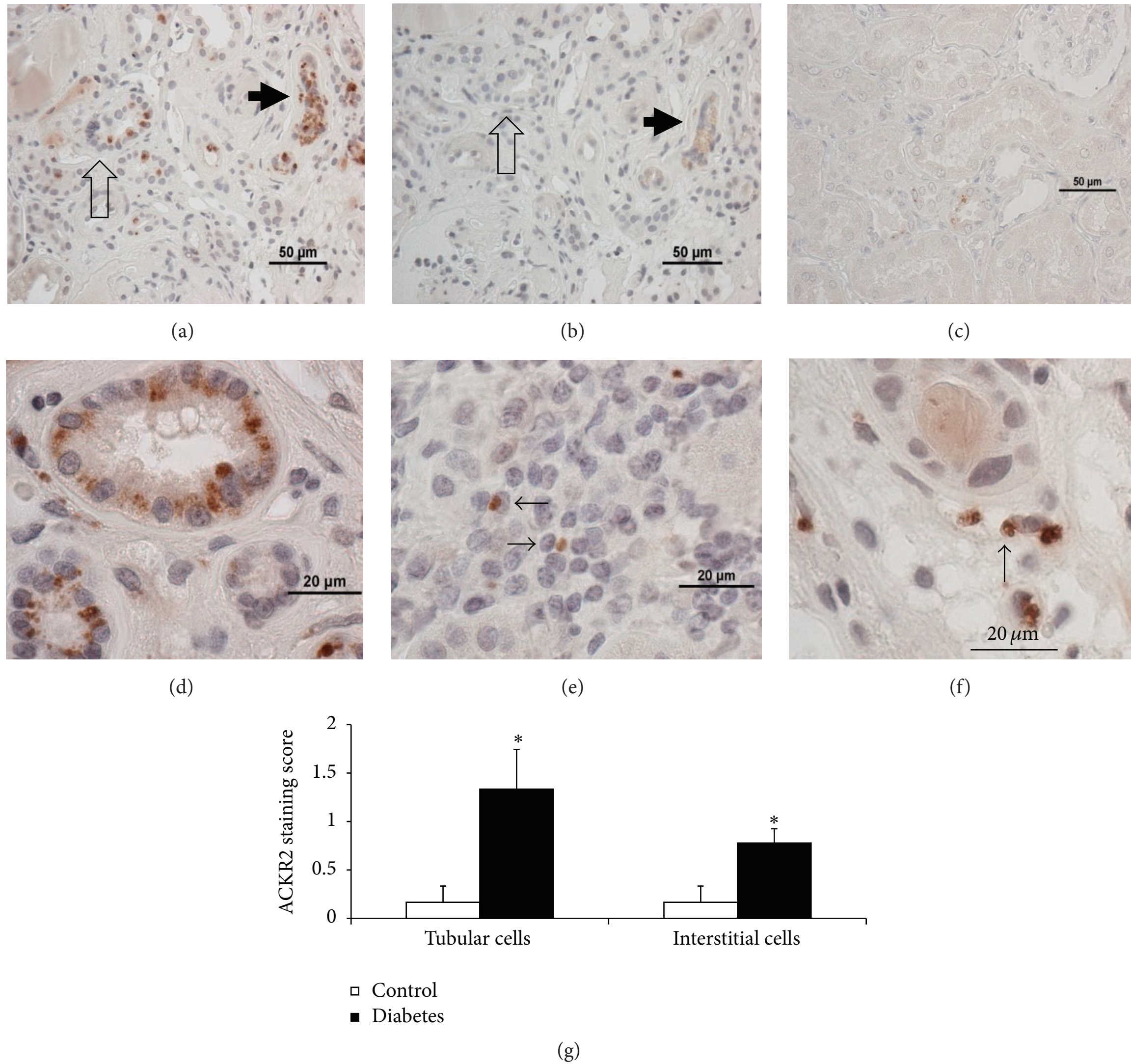

FIGURE 6: Increased ACKR2 protein in diabetic human kidney sections stained with rat monoclonal antibody to human ACKR2. (a) Positive ACKR2 staining in diabetic kidney. Strongest staining in tubules (arrows) especially in a collapsed (arrow) tubule. (b) Minimal staining is seen on a serial section without primary antibody. The arrows indicate the same 2 tubules in images (a) and (b). (c) Sparse ACKR2 staining in a nondiabetic section. (d) At higher magnification granule-like deposits of ACKR2 can be seen in cytoplasm of proximal tubular epithelial cells in diabetic kidney. In the interstitial space ACKR2 staining is also visible in diabetic kidney monocytes (e) and endothelial cells (f). (g) Semiquantitative scoring of ACKR2 staining by a scorer blind to sample identity. Scores for proximal tubule and interstitial cells are higher in diabetic than nondiabetic samples. $^{*}p < 0.05$ by t-test, $n = 9$ diabetic and 6 nondiabetic samples.

Surprisingly little information is available for the ACKR2 chemokine scavenger receptor in the kidney, and only previous study indicated that the level of ACKR2 RNA in mouse kidney is low [15]. The current study also found low expression of ACKR2 RNA in normal kidney, approximately fiftyfold lower than in lung. We further observed a tendency for diabetes to increase ACKR2 RNA expression in OVE mouse kidney.

To determine if the ACKR2 RNA results indicate that diabetes alters ACKR2 protein immunohistochemistry studies were performed on human tissue since only an anti-human ACKR2 antibody has been validated [18, 29, 30]. Diabetic kidneys had significantly stronger ACKR2 staining in tubule and interstitial cells. Staining increased primarily in proximal tubule cells and in tubule cells that were too abnormal to distinguish as proximal or distal (Figure 6(a)). ACKR2 positive interstitial cells seen in diabetic samples appeared to be a mix of infiltrating monocytes and endothelial cells which could belong to blood or lymphatic vessels. The ACKR2 positive cell profile in kidney was not unusual. Positive stromal cells were expected, since ACKR2 staining in other organs has been reported for monocytes, lymphocytes, dendritic cells, and endothelial cells. Increased infiltration of inflammatory cells is common in diabetic kidneys [7, 8, 32]. Tubule cell staining for ACKR2 is unsurprising considering ACKR2 has been shown in parenchymal cells of several

organs: ACKR2 antibodies stain epidermis in psoriatic skin [33], syncytiotrophoblast cells of placenta [18], and breast cancer cells [34]. The absence of ACKR2 staining in diabetic glomeruli indicates that direct actions of ACKR2 are limited to the tubular and interstitial portions of the diabetic kidney.

The primary finding of this study was that ACKR2 deletion dramatically reduced DN. The reduction of albuminuria in OVE-ACKR2 mice was significant at the earliest age tested, two months. As OVE mice aged DN progressed and the protection by ACKR2 KO became more striking. At 6 months ACKR2 deletion produced a greater reduction in diabetic albuminuria. In addition several markers demonstrated reduced inflammation in OVE-ACKR2 kidneys compared to OVE kidneys. Histologically this was indicated by decreased leukocyte infiltration and less fibrosis. Gene expression data demonstrated that absence of ACKR2 prevented activation of multiple molecular pathways involved in immune or inflammatory processes in kidneys of diabetic mice. The finding of such potent renal protection from diabetes by deletion of ACKR2 was contradictory to our expectation, which was that deletion of ACKR2 would exacerbate DN by increasing renal inflammation. This expectation was based on the damage inflammation produces in DN and the anti-inflammatory potency of ACKR2 as a scavenger of proinflammatory chemokines. In several studies manipulation of ACKR2 levels modified tissue inflammation in a manner that would be predicted based on anti-inflammatory potency of ACKR2 as a chemokine scavenger: this was shown in experimental models of colitis and psoriasis, where deletion of ACKR2 increased colon [21] or skin [17] inflammation, and in inflamed NOD mouse islets where transgenic overexpression of ACKR2 reduced local islet inflammation [35].

Mechanisms to explain protection from DN by deletion of ACKR2 are not obvious. Protection was not due to reduced OVE diabetes since hyperglycemia was equivalent in OVE and OVE-ACKR2 mice (Figure 1). In considering potential mechanisms of protection by ACKR2 KO it needs to be considered that this is not the first such report. Unexpected protection by deletion of ACKR2 has been reported to reduce pathology of several inflammatory diseases: ACKR2 deletion inhibits spinal cord inflammation and autoimmune encephalomyelitis [36], reduces susceptibility and symptoms of dextran sulfate-induced colitis [37], and reduces airway reactivity in allergen-induced airway disease [19]. In addition to these inflammatory disease models, KO of host ACKR2 can suppress transplant graft rejection [38, 39]. The unexpected but repeated finding of beneficial effects of ACKR2 deletion in multiple disease models indicates that our anti-inflammatory concept of ACKR2 was overly simplistic and the chemokine scavenging properties of ACKR2 may produce complex and not purely anti-inflammatory results. For example, deletion of ACKR2 releases chemokines that promote increased production of immunosuppressive monocytes [39] that reduce graft-versus-host disease. During chronic DN progression complex and changing interactions occur between the immune system and the kidney. At this time, the underlying molecular mechanisms involved in diabetic kidney disease are not clear. It is possible that kidney damage initiated by hyperglycemia is more efficiently cleared in ACKR2 mice. More rapid damage removal decreases the chances of developing chronic inflammation. Despite uncertainty about the mechanism, the strength of protection produced by elimination of ACKR2 indicates that it has a key role in the pathology which needs to be dissected at a finer level.

In *summary*, we found that deletion of the ACKR2 gene produced a dramatic reduction in albuminuria and renal inflammation in the OVE diabetic mouse without decreasing diabetes. In human samples diabetes increased the expression of ACKR2 protein in tubule cells, leukocytes, and endothelial cells.

Conflict of Interests

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

Acknowledgments

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Review Article

A Systems Biology Overview on Human Diabetic Nephropathy: From Genetic Susceptibility to Post-Transcriptional and Post-Translational Modifications

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Diabetic nephropathy (DN), a microvascular complication occurring in approximately 20–40% of patients with type 2 diabetes mellitus (T2DM), is characterized by the progressive impairment of glomerular filtration and the development of Kimmelstiel-Wilson lesions leading to end-stage renal failure (ESRD). The causes and molecular mechanisms mediating the onset of T2DM chronic complications are yet sketchy and it is not clear why disease progression occurs only in some patients. We performed a systematic analysis of the most relevant studies investigating genetic susceptibility and specific transcriptomic, epigenetic, proteomic, and metabolomic patterns in order to summarize the most significant traits associated with the disease onset and progression. The picture that emerges is complex and fascinating as it includes the regulation/dysregulation of numerous biological processes, converging toward the activation of inflammatory processes, oxidative stress, remodeling of cellular function and morphology, and disturbance of metabolic pathways. The growing interest in the characterization of protein post-translational modifications and the importance of handling large datasets using a systems biology approach are also discussed.

1. Introduction

DN is an endemic complication of diabetes and the first cause of ESRD worldwide. The contributing causes of DN pathogenesis and progression are still poorly understood but chronic hyperglycemia and high blood pressure represent the main risk factors for disease onset.

Hemodynamic and Biochemical Background. In the early stages of DN, high systemic blood pressure usually determines an increase of the intraglomerular pressure and glomerular filtration rate (GFR) which results in glomerular hyperfiltration [1]. From the biochemical point of view, hyperglycemia *per se* sustains the accumulation of advanced glycation end products (AGEs), altering the electronegativity of the cell; additionally AGEs bind proteins of the extracellular matrix (ECM) inhibiting their degradation. AGEs accumulation can induce an increased production of reactive oxygen species (ROS) and a transcriptional activation of different proinflammatory and profibrotic molecules, including TGF-beta [2, 3]. The high glucose-mediated induction of TGF-beta and the central role of this growth factor in DN progression represent the few defining constants in the pathogenesis of DN [4].

Clinical and Histological Hallmarks of DN. The earliest clinical signs of DN include a slight but persistent urinary excretion of albumin (microalbuminuria) and a temporary increase of the glomerular filtration rate (GFR). These clinical signs, along with the presence of hyperglycemia, are often considered sufficient indicators of DN [5, 6]. Today, extensive evidence shows that DN is not the only type of renal damage that can be

found in diabetic patients [7, 8] and kidney biopsy, although highly invasive, remains the diagnostic gold standard. The histological hallmarks of DN include hyperproliferation of the mesangial cells, thickening of the glomerular basement membrane (GBM), podocyte effacement, tubulointerstitial fibrosis, and nodular accumulations of ECM (Kimmelstiel-Wilson lesions) in the glomerulus [9].

Given the high prevalence of type 2 diabetes (T2D) and the diagnostic limitations currently associated with kidney biopsy, there is an impending need for new, accurate, and easily accessible biomarkers of disease.

In this review we will try to outline a system biology overview on DN by recapitulating the main annotations obtained at different levels of molecular investigation. Only those studies investigating human samples will be described; the murine models of DN in fact, although undergoing albuminuria, mesangial expansion, and podocyte loss, do not develop severe glomerulosclerosis and tubulointerstitial fibrosis [10]. Also, as substantial differences exist in the etiology and prevalence of type 1 and type 2 DN, the articles discussed in this paper apply to DN secondary to type 2 diabetes (T2DN). As an exception, works describing biomarkers of kidney damage in T1D that have been further validated in T2DM and *vice versa* and those reporting potential prognostic biomarkers, because of their particular importance in predicting the progression of renal damage, have been also discussed in the present work. All the annotations discussed in this review are also listed in Tables 1, 2, 3, 4, and 5, categorized according to whether they summarize the genetic and transcriptomic signature of coding or noncoding RNA molecules and the epigenetic proteomic and metabolomic markers, respectively.

2. Genetic Profiling of DN

Genetic variation is present under different forms in the human genome, ranging from single nucleotide polymorphisms (SNPs) to large, structural, chromosomal rearrangements. Today we know that genetic variation infers disease susceptibility and collective effort aims at identifying the precise loci for DN susceptibility. Different methodological strategies can be used to characterize the genetic risk for a disease, either targeted or genome-wide, according to whether *a priori* hypothesis of the candidate regions for disease susceptibility exists. In genome-wide association studies (GWAS), for instance, the whole genome is screened for new, previously uncharacterized single nucleotide polymorphisms (SNPs).

Prior to the development of the modern high-throughput technologies such as chip-based microarray analysis and next-generation sequencing, the inheritance of disease susceptibility was investigated through genetic linkage in families. Basically, individuals within the same families were sequenced for a collection of genetic SNPs in order to identify those SNPs segregating with the disease. This approach led to the identification of many variants responsible for disease susceptibility but it proved mostly suitable for the study of single gene disorders. For complex, common complications like T2D in fact, progression is very likely driven by multiple alleles simultaneously, each having a small correlation to disease progression if inherited individually. This implies that a big population needs to be genotyped in order to detect the common variants responsible for the increased genetic risk.

In the field of DN, there is extensive evidence for genetic contribution to disease susceptibility. In 1989, Seaquist et al. showed that diabetic siblings of patients with DN were more at risk for developing DN compared to diabetic siblings of diabetic patients without proteinuria [99]; epidemiologic studies also indicate that the prevalence of DN varies among ethnic groups [100]. These observations, along with the consideration that only a subset of patients with diabetes develops DN, drove the search for the genetic determinants of DN susceptibility.

One of the most consistent annotations in the field is probably the genetic variation on chromosome 18. In 2002, a family-based linkage analysis performed in T2DN Turkish families and affected sibling pairs of Pima Indians reported a strong evidence for the localization of a DN susceptibility locus mapping to chromosome 18q22.3-23 [12]. Researchers were not able to pinpoint the precise susceptibility gene but the same locus was also detected in a T2DN African American population [11]. Later studies on chromosome 18 led to the identification of a susceptibility marker within the carnosine dipeptidase 1 (CNDP1) gene, and it was also described how the shortest allelic form of the CNDP1 gene was more common in the absence of nephropathy [15]. The CNDP1 gene encodes the secreted enzyme serum carnosinase that degrades carnosine, a protein controlling the formation of AGE molecules [101]. As previously discussed, AGE's accumulation is a phenotypic sign of DN. Similar results were obtained in a meta-analysis study when investigating a multiethnic population with T2D-ESRD [16]; a recently published meta-analysis confirmed the association of the carnosinase D18S880 microsatellite polymorphism with DN susceptibility in a T2D Caucasian population although no significant association with T1DN could be found [17].

In a very recent candidate-gene driven study, Palmer et al. performed a genotyping of several SNPs across 22 DN candidate genes in a large cohort of African Americans with T2D and ESRD. After adjustment for the APOL1 G1/G2 alleles, known to be associated with nondiabetic ESRD in this population, the most significant signals were observed downstream of the CNDP1 gene, at chimerin 2 (CHN2) locus and within angiotensin II receptor type 1 (AGTR1) gene [13]. In another work, to investigate the impact of oxidative stress on disease initiation, the polymorphic variants of 7 genes involved in the antioxidant defense were evaluated: SOD2, p22 phox, CAT, MPO, GSTP1, GSTT1, and GSTM1. Despite the commonly recognized link between oxidative stress and diabetes, authors claim that no association could be found in Caucasian T2D patients [102].

In one of the first DN genome-wide genotyping studies, authors reported the engulfment and cell motility 1 (ELMO1) gene on chromosome 7p as a likely candidate for disease susceptibility in a Japanese patients cohort with T2D [19]. In a cellular system engineered to overexpress ELMO1, they furthermore observed increased expression of extracellular matrix (ECM) protein genes and decreased expression

Table 1: Genetic markers. Collection of significant genetic markers, listed alphabetically.

Nearest gene(s)	Variant	Ethnicity	Diabetes type	Assay type	Potential value of biomarker	References
18q	D18S1364	African American	T2D	Linkage analysis	Diagnostic	[11]
18q22.3-23	D18S43/D18S50	Turkish	T2D	Linkage analysis	Diagnostic	[12]
18q22.3-23	D18S43/D18S50	Pima Indians	T2D	Linkage analysis	Diagnostic	[12]
7p	D7S3051	African American	T2D	Linkage analysis	Diagnostic	[11]
AGTR1	rs12695897	African American	T2D	Candidate based genotyping	Diagnostic	[13]
APOL3	rs16996381	African American	T2D	GWAS	Diagnostic	[14]
AUH	rs773506	African American	T2D	GWAS	Diagnostic	[14]
C12orf66/TMEM5	rs11175885	African American	T2D	GWAS	Diagnostic	[14]
C6orf167	rs3822908	African American	T2D	GWAS	Diagnostic	[14]
C6orf191/ARHGAP18	rs208865	African American	T2D	GWAS	Diagnostic	[14]
CHN2	rs2057737	African American	T2D	Candidate based genotyping	Diagnostic	[13]
CHN2	rs3729621	African American	T2D	Candidate based genotyping	Diagnostic	[13]
CHN2	rs3793313	African American	T2D	Candidate based genotyping	Diagnostic	[13]
CNDP1	D18S880	European	T1D/T2D	Candidate based genotyping	Diagnostic	[15]
CNDP1	D18S880	Multiethnic	T2D	Meta-analysis	Diagnostic	[16]
CNDP1	rs4892249	African American	T2D	Candidate based genotyping	Diagnostic	[13]
CNDP1	rs6566815	African American	T2D	Candidate based genotyping	Diagnostic	[13]
CNDP1	D18S880	Caucasian	T2D	Meta-analysis	Diagnostic	[17]
ELMO1	rs741301	Asian	T2D	Meta-analysis	Diagnostic	[16]
ELMO1	rs11769038	Caucasian	T1D	GWAS	Diagnostic	[18]
ELMO1	rs1882080	Caucasian	T1D	GWAS	Diagnostic	[18]
ELMO1	rs2041801	Caucasian	T1D	GWAS	Diagnostic	[18]
ELMO1	rs7785934	Caucasian	T1D	GWAS	Diagnostic	[18]
ELMO1	Intron 18 + 9170 (A/G)	Japanese	T2D	GWAS	Diagnostic	[19]
FRMD3	rs1535753	African American	T2D	GWAS	Diagnostic	[20]
FRMD3	rs2378658	African American	T2D	GWAS	Diagnostic	[20]
FRMD3	rs942278	African American	T2D	GWAS	Diagnostic	[20]
FRMD3	rs942280	African American	T2D	GWAS	Diagnostic	[20]
FRMD3	rs942283	African American	T2D	GWAS	Diagnostic	[20]

Table 1: Continued.

Nearest gene(s)	Variant	Ethnicity	Diabetes type	Assay type	Potential value of biomarker	References
FRMD3	rs1888747	European	T2D	Candidate based genotyping	Diagnostic	[21]
GRIK2	rs7760831	African American	T2D	GWAS	Diagnostic	[14]
GRIP1/CAND1	rs11176482	African American	T2D	GWAS	Diagnostic	[14]
GRIP1/CAND1	rs2904532	African American	T2D	GWAS	Diagnostic	[14]
LIMK2	rs2106294	African American	T2D	GWAS	Diagnostic	[14]
LIMK2	rs4820043	African American	T2D	GWAS	Diagnostic	[14]
MSRB3/HMGA2	rs2358944	African American	T2D	GWAS	Diagnostic	[14]
MYH9	rs735853	African American	T2D	GWAS	Diagnostic	[14]
NAV3	rs12302041	African American	T2D	GWAS	Diagnostic	[14]
ND	rs1978243	African American	T2D	GWAS	Diagnostic	[14]
ND	rs4260465	African American	T2D	GWAS	Diagnostic	[14]
ND	rs7697691	African American	T2D	GWAS	Diagnostic	[14]
OR2L13	rs10888287	African American	T2D	GWAS	Diagnostic	[14]
PVT1	rs2648875	Pima Indians	T2D	GWAS	Diagnostic	[22]
PVT1	rs13447075	European	T1D	Candidate based genotyping	Diagnostic	[23]
RNF185	rs1034589	African American	T2D	GWAS	Diagnostic	[14]
RPS12	rs7769051	African American	T2D	GWAS	Diagnostic	[14]
RPS12	rs9493454	African American	T2D	GWAS	Diagnostic	[14]
SASH1	rs6930576	African American	T2D	GWAS	Diagnostic	[14]
SFI1	rs5749286	African American	T2D	GWAS	Diagnostic	[14]
SLC10A7/LSM6	rs891382	African American	T2D	GWAS	Diagnostic	[14]
TPM1	rs6494387	African American	T2D	GWAS	Diagnostic	[14]
UNC5C	rs11730446	African American	T2D	GWAS	Diagnostic	[14]

of matrix metalloproteinases [103]. The same susceptibility locus was also identified in a T1DN Caucasian cohort [18]. Finally, recent data from a meta-analysis study suggests the ELMO1 association with DN exclusively in the T2D Asian subgroup [16].

In a population of Pima Indians with T2D, the GWAS of over 100,000 SNPs led to the identification of several loci with significant association for ESRD susceptibility, with the strongest signal located in the intronic region of the of PVT1 gene [22]. Some of these findings were also replicated in an ethnically different population with T1D [23].

In a GWAS performed on a large cohort of African Americans with T2D and ESRD, five gene regions with evidence of association with DN were detected, nominally, SASH1, RPS12, AUH, MSRB3-HMGA2, and LIMK2-SFI1. Some of these SNPs however were later found to contribute to all-cause ESRD [14].

In order to establish a comprehensive, well-defined DNA biobank for the genotyping of DN in T1D in particular, the Genetics of Kidneys in Diabetes (GoKinD) study was undertaken [104]. The first results of this genome-wide scan were reported by Pezzolesi et al. in 2009. Authors claimed that although no SNP achieved genome-wide significance, strong association was found near the 4.1 protein ezrin, radixin, and moesin [FERM] domain containing 3 (FRMD3) locus and near the cysteinyl-tRNA synthetase (CARS) locus [105]. Further studies confirmed the 9q21.32 region (upstream of FRMD3) as a susceptibility locus for T2DN in several unrelated study populations [20, 21].

Despite all the effort currently invested into this field of research, at present it is still impossible to predict those diabetic patients with a higher risk for developing DN. Indeed, in almost all the studies published so far on DN susceptibility, diagnosis was based almost exclusively on the presence of hyperglycemia and proteinuria; therefore, it is not possible to exclude that the inconsistencies among the findings could be linked to a misclassification of the renal damage in the diabetic population.

The genetic markers cited in this paper are also summarized in Table 1.

Table 2: Gene expression markers. Collection of coding RNA transcripts showing deregulation in DN. List is ordered alphabetically. IHC: immunohistochemistry. SAGE: serial analysis of gene expression; NGS: next-generation sequencing.

mRNA transcript	Sample type	Tissue compartment	Expression	Diabetes type	Assay type	Potential value of biomarker	References
ABCA1	Kidney	Whole	Down	T2D	qPCR	Diagnostic/prognostic	[24]
ABCG1	Kidney	Whole	Down	T2D	qPCR	Diagnostic/prognostic	[24]
ACE	Kidney	Whole	Up	T2D	qPCR	Diagnostic	[25]
ACE	Kidney	Glomerular	Up	T2D	qPCR	Diagnostic	[26]
ACE	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[27]
ACE2	Kidney	Glomerular	Down	T2D	qPCR	Diagnostic	[26]
ACTN4	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[28]
ACTN4	Urine	Sediment	Up	T2D	qPCR	Diagnostic/prognostic	[29]
ACTN4	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[27]
ANGPTL2	Kidney	Glomerular	Up	T2D	qPCR/IHC	Diagnostic	[30]
ANKRD56	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[31]
apoE	Kidney	Whole	Down	T2D	qPCR	Diagnostic/prognostic	[24]
AQP1	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[32]
ATG5	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[33]
ATG7	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[34]
ATG8	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[33]
B2M	Kidney	Tubulointerstitial	Up	T1D/T2D	Array/qPCR	Diagnostic/prognostic	[35]
B7-1	Kidney	Whole	Up	T2D	qPCR	Diagnostic	[36]
BECN1	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[33]
BECN1	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[34]
BMP2	Kidney	Glomerular	Down	T2D	Array	Diagnostic	[32]
C3	Kidney	Glomerular	Up	ND	Array	Diagnostic	[37]
CAPN3	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[32]
CCL2	Kidney	Tubulointerstitial	Up	T2D	qPCR/IHC	Diagnostic	[38]
CCL5/RANTES	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic/prognostic	[35]
CCR5	Kidney	Tubulointerstitial	Up	T2D	qPCR/IHC	Diagnostic	[38]
CD2AP	Urine	Sediment	Up	T2D	qPCR	Diagnostic/prognostic	
CD36	Kidney	Whole	Up	T2D	qPCR	Diagnostic/prognostic	[24]
CD68	Kidney	Tubulointerstitial	Up	T2D	qPCR/IHC	Diagnostic	[38]
CDH2	Urine	Sediment	Up	T2D	qPCR	Diagnostic/prognostic	[27]
CLIC5	Kidney	Glomerular	Down	ND	Array/IHC	Diagnostic	[37]
COL1A2	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
COL1A2	Kidney	Glomerular	Up	ND	Array	Diagnostic	[37]
COL1A2	Kidney	Tubular	Up	ND	Array	Diagnostic	[37]
COL3A1	Kidney	Tubular	Up	ND	Array	Diagnostic	[37]
COL4A1	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
COL4A1	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[27]
COL4A2	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
COL6A3	Kidney	Glomerular	Up	ND	Array	Diagnostic	[37]
COL8A1	Kidney	Glomerular/ tubulointerstitial	Up	T2D	qPCR/IHC	Diagnostic	[40]
COL8A2	Kidney	Glomerular/ tubulointerstitial	Up	T2D	qPCR/IHC	Diagnostic	[40]
CTGF	Kidney	Glomerular	Down	T2D	Array/qPCR/IHC	Diagnostic	[41]
CXCL10/IP10	Kidney	Tubulointerstitial	Up	T1D/T2D	Array/qPCR	Diagnostic/prognostic	[35]
CXCL16	Kidney	Whole	Down	T2D	qPCR	Diagnostic/prognostic	[24]

Table 2: Continued.

mRNA transcript	Sample type	Tissue compartment	Expression	Diabetes type	Assay type	Potential value of biomarker	References
CXCL6	Kidney	Glomerular	Up	ND	Array	Diagnostic	[37]
DKK3	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[42]
EDN1	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic/prognostic	[35]
EGF	Kidney	Tubulointerstitial	Down	T1D/T2D	qPCR/IHC	Diagnostic/prognostic	[39]
ENTPD8	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[31]
FAT1	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[27]
FGF-2	Kidney	Tubulointerstitial	Up	T2D	qPCR/IF	Diagnostic	[43]
FN1	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
FOXO1	Kidney	Whole	Down	T2D	qPCR/IHC	Diagnostic	[33]
FOXO3A	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[33]
FSP1	Kidney	Glomerular	Up	T2D	qPCR/ISH	Diagnostic/prognostic	[44]
GREM1	Kidney	Whole	Up	T2D	qPCR/ISH/IHC	Diagnostic	[45]
GREM1	Kidney	Whole	Up	T2D	qPCR/ISH	Diagnostic/prognostic	[46]
HDAC2	Kidney	Whole	Up	T2D	qPCR/IHC	Diagnostic/prognostic	[34]
HDAC4	Kidney	Whole	Up	T2D	qPCR/IHC	Diagnostic/prognostic	[34]
HDAC5	Kidney	Whole	Up	T2D	qPCR/IHC	Diagnostic/prognostic	[34]
HES1	Kidney	Whole	Up	T2D	qPCR/ISH	Diagnostic	[46]
HLA-A	Kidney	Tubulointerstitial	Up	T1D/T2D	Array/qPCR	Diagnostic/prognostic	[35]
HLA-B	Kidney	Tubulointerstitial	Up	T1D/T2D	Array/qPCR	Diagnostic/prognostic	[35]
HSPA5	Kidney	Tubulointerstitial	Up	T2D	Array/qPCR	Diagnostic	[47]
Hyaluronoglucosidase	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[32]
HYOU1	Kidney	Tubulointerstitial	Up	T2D	Array/qPCR	Diagnostic	[47]
IFNB1	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic/prognostic	[35]
IGF-1	Kidney	Glomerular	Down	T2D	Array	Diagnostic	[32]
IGFBP-2	Kidney	Glomerular	Down	T2D	Array	Diagnostic	[32]
IGH	Kidney	Glomerular	Up	ND	Array	Diagnostic	[37]
IGH	Kidney	Tubular	Up	ND	Array	Diagnostic	[37]
IGL	Kidney	Tubular	Up	ND	Array	Diagnostic	[37]
IHG-1	Kidney	Tubulointerstitial	Up	T2D	ISH	Diagnostic	[48]
IL6	Kidney	Tubulointerstitial	Up	T2D	qPCR/IHC	Diagnostic	[38]
IRS2	Kidney	Tubulointerstitial	Up	T2D	ISH	Diagnostic	[49]
JAG1	Kidney	Whole	Up	T2D	qPCR/ISH	Diagnostic	[46]
JAK2	Kidney	Glomerular/ tubulointerstitial	Up	T2D	Array/qPCR/IHC	Diagnostic/prognostic	[50]
LC3	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[33]
LC3	Kidney	Whole	Down	T2D	qPCR	Diagnostic	[34]
LDLR	Kidney	Whole	Up	T2D	qPCR	Diagnostic/prognostic	[24]
LEF1	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[42]
LOX1	Kidney	Whole	Up	T2D	qPCR	Diagnostic/prognostic	[24]
MMP14	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
MMP2	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
MMP7	Kidney	Tubulointerstitial	Up	ND	Array/qPCR/IHC	Diagnostic	[51]
MMP7	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
MRP8	Kidney	Glomerular	Up	T2D	qPCR/IHC	Diagnostic/prognostic	[52]
NOTCH3	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[27]
NPHS1	Kidney	Glomerular	Down	T2D	Array	Diagnostic	[41]
NPHS1	Kidney	Glomerular	Down	T2D	Array/IHC	Diagnostic	[32]
NPHS1	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[53]

Table 2: Continued.

mRNA transcript	Sample type	Tissue compartment	Expression	Diabetes type	Assay type	Potential value of biomarker	References
NPHS1	Kidney	Whole	Down	T2D	ISH	Diagnostic/prognostic	[54]
NPHS1	Urine	Sediment	Up	T2D	qPCR	Diagnostic/prognostic	[28]
NPHS1	Kidney	Glomerular	Down	ND	Array	Diagnostic	[37]
NPHS2	Kidney	Glomerular	Down	T2D	Array	Diagnostic	[41]
NPHS2	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[53]
NPHS2	Urine	Sediment	Up	T2D	qPCR	Diagnostic/prognostic	[28]
NPHS2	Kidney	Glomerular	Down	ND	Array/IHC	Diagnostic/prognostic	[37]
NPHS2	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[29]
NRP1	Kidney	Glomerular	Down	T2D	qPCR	Diagnostic	[55]
NRP2	Kidney	Glomerular	Down	T2D	qPCR	Diagnostic	[55]
OPG	Kidney	Tubulointerstitial	Up	T2D	Array/qPCR	Diagnostic/prognostic	[56]
PDGF-A	Kidney	Whole	Up	T2D	qPCR/IHC	Diagnostic	[57]
PDGF-B	Kidney	Whole	Up	T2D	qPCR/IHC	Diagnostic	[57]
PECAM-1	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[32]
PKCα	Kidney	Glomerular	Up	T2D	qPCR/IHC	Diagnostic	[58]
PLA2R1	Kidney	Glomerular	Down	ND	Array	Diagnostic	[37]
PLCE1	Kidney	Glomerular	Down	ND	Array	Diagnostic	[37]
PODXL	Kidney	Glomerular	Down	ND	Array	Diagnostic	[37]
PODXL	Urine	Sediment	Up	T2D	qPCR	Diagnostic/prognostic	[29]
PRKCB	Kidney	Whole	Up	T2D	qPCR	Diagnostic	[59]
PTGDS	Kidney	Glomerular	Down	ND	Array	Diagnostic	[37]
ROBO2	Kidney	Glomerular	Down	T2D	qPCR	Diagnostic	[60]
SMPDL3b	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[61]
STAT1	Kidney	Whole	Up	T2D	qPCR/IHC	Diagnostic	[33]
SYNPO	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[28]
SYNPO	Kidney	Glomerular	Down	ND	Array	Diagnostic	[37]
SYNPO	Urine	Sediment	Up	T2D	qPCR	Diagnostic/prognostic	[29]
SYNPO	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[27]
TIMP1	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
TIMP1	Urine	Sediment	Down	T2D	qPCR	Diagnostic	[27]
TIMP3	Kidney	Whole	Down	T2D	qPCR/IHC	Diagnostic	[33]
TIMP3	Kidney	Tubulointerstitial	Up	T1D/T2D	qPCR	Diagnostic	[39]
TIPE2	Kidney	Whole	Up	T2D	qPCR/WB	Diagnostic	[62]
TLR4	Kidney	Glomerular/ tubulointerstitial	Up	T2D	qPCR/IHC	Diagnostic/prognostic	[38]
TNFAIP8	Kidney	Whole	Up	T2D	qPCR/WB	Diagnostic	[62]
TRAIL	Kidney	Tubulointerstitial	Up	T2D	Array/IHC/qPCR	Diagnostic/prognostic	[56]
TWIST1	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[27]
UII	Kidney	Whole	Up	T2D	qPCR/IHC	Diagnostic	[63]
UT	Kidney	Whole	Up	T2D	qPCR	Diagnostic	[63]
VCAM1	Kidney	Tubulointerstitial	Up	T1D/T2D	Array/qPCR	Diagnostic/prognostic	[35]
VEGF	Kidney	Glomerular	Down	T2D	Array/qPCR/IHC	Diagnostic	[41]
VEGF	Kidney	Glomerular	Down	T2D	Array/IHC	Diagnostic/prognostic	[32]
VEGF	Kidney	Glomerular	Down	T2D	qPCR	Diagnostic/prognostic	[64]
VEGF	Kidney	Whole	Up	T2D	ISH/IHC	Diagnostic	[65]
VEGF	Kidney	Tubulointerstitial	Down	T1D/T2D	qPCR/IHC	Diagnostic/prognostic	[39]
WNT1	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[42]
WNT16	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[42]

Table 2: Continued.

mRNA transcript	Sample type	Tissue compartment	Expression	Diabetes type	Assay type	Potential value of biomarker	References
WNT2B	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[42]
WNT4	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[42]
WNT6	Kidney	Glomerular	Up	T2D	Array	Diagnostic	[42]
WT1	Kidney	Glomerular	Down	T2D	Array/IHC	Diagnostic	[41]
WT1	Urine	Sediment	Up	T2D	qPCR	Diagnostic	[28]
WT1	Kidney	Glomerular	Down	ND	Array	Diagnostic/prognostic	[37]
XBP1	Kidney	Tubulointerstitial	Up	T2D	Array/qPCR	Diagnostic	[47]

Table 3: Noncoding RNA markers. Collection of noncoding RNA transcripts deregulated in DN samples. List is ordered alphabetically.

miRNA transcript	Sample type	Expression	Assay type	Diabetes type	Potential value of biomarker	References
hsa-miR-1205	Mesangial cells	Up	qPCR	—	Descriptive	[66]
hsa-miR-129	Human mesangial cells	Up	Array/qPCR	—	—	[67]
hsa-miR-130a	Urinary exosomes	Up	qPCR	T1D	Diagnostic	[68]
hsa-miR-145	Urinary exosomes	Up	qPCR	T1D	Diagnostic	[68]
hsa-miR-146a	Kidney	Up	Array/qPCR	T2D	Diagnostic	[69]
hsa-miR-15	Urinary sediment	Down	qPCR	—	Diagnostic/prognostic	[70]
hsa-miR-155	Urinary exosomes	Down	qPCR	T1D	Diagnostic/prognostic	[68]
hsa-miR-155	Kidney	Up	Array/qPCR	T2D	Prognostic	[69]
hsa-miR-188-3p	Urine	Down	qPCR	T1D	Prognostic	[71]
hsa-miR-1913	Urine	Up	qPCR	T1D	Prognostic	[71]
hsa-miR-192	Kidney	Down	qPCR/ISH	—	Diagnostic/prognostic	[72]
hsa-miR-192	Human mesangial cells	Up	Array	—	—	[67]
hsa-miR-192	Urinary sediment	Down	qPCR	—	Diagnostic/prognostic	[73]
hsa-miR-21	Kidney	Up	qPCR	T2D	Diagnostic	[74]
hsa-miR-214-3p	Urine	Up	qPCR	T1D	Prognostic	[71]
hsa-miR-221-3p	Urine	Down	qPCR	T1D	Prognostic	[71]
hsa-miR-29a	Urine	Up	qPCR	T2D	Prognostic	[75]
hsa-miR-323b-5p	Urine	Down	qPCR	T1D	Prognostic	[71]
hsa-miR-337	Human mesangial cells	Up	Array/qPCR	—	—	[67]
hsa-miR-373-5p	Urine	Up	qPCR	T1D	Prognostic	[71]
hsa-miR-377	Human mesangial cells	Up	Array/qPCR	—	—	[67]
hsa-miR-424	Urinary exosomes	Down	qPCR	T1D	Prognostic	[68]
hsa-miR-429	Urine	Up	qPCR	T1D	Prognostic	[71]
hsa-miR-524-5p	Urine	Down	qPCR	T1D	Prognostic	[71]
hsa-miR-638	Urine	Up	qPCR	T1D	Prognostic	[71]
hsa-miR-765	Urine	Up	qPCR	T1D	Prognostic	[71]
hsa-miR-92b-5p	Urine	Up	qPCR	T1D	Prognostic	[71]
let-7a	Whole blood	Down	Array/qPCR	T2D	Diagnostic	[76]
PVT1 (lncRNA)	Mesangial cells	Up	qPCR	—	Descriptive	[66]

3. Transcriptome Profiling of DN

The transcriptome represents the part of genome that is transcribed and includes both coding and noncoding RNA molecules. When studying the transcriptome, as for genetic studies, either targeted or genome-wide approaches can be used. RNA-sequencing (RNA-seq), arrays, and quantitative PCR (qPCR) are the techniques employed routinely to assess RNA expression. qPCR is very sensitive and even subtle changes can be detected precisely; arrays on the other hand are very high-throughput but also less sensitive. RNA-seq takes advantage of the recent next-generation sequencing platforms and it has rapidly become the method of choice for transcriptome profiling. The main advantages of RNA-Seq are its very high resolution (down to a single nucleotide), its potential to detect novel transcripts, its ability to measure

either primary transcripts or spliced mature mRNAs. Given the plethora of gene expression data available in the literature, only the research on DN kidney tissue or urine will be discussed. All the coding and noncoding RNA markers cited in this paper are also summarized in Tables 2 and 3, respectively.

3.1. Coding RNA Studies. The first transcriptomic signature of DN kidney was published in 2004. Using an array-based approach, Baelde et al. assayed the glomerular gene expression profile of T2DN and morphologically normal, nondiabetic kidneys. The results of this genome-wide analysis indicated that 96 genes were upregulated in T2DN, including aquaporin 1 (AQP1), calpain 3 (CAPN3), hyaluronoglucosidase, and platelet/endothelial cell adhesion molecule (PECAM-1). Over 500 genes were downregulated, including bone morphogenetic protein 2 (BMP2), vascular endothelial growth factor (VEGF), fibroblast growth factor 1 (IGF-1), insulin-like growth factor binding protein 2 (IGFBP-2), and nephrin. In the same manuscript, authors confirmed reduced expression of VEGF and nephrin in renal biopsy specimens from additional DN patients at both the protein and RNA levels [32].

To explain the existing inconsistencies between human and murine progressive DN, microdissected biopsies from controls, early and progressive T2DN patients underwent global gene expression profiling through microarray hybridization. Preliminary results, later confirmed using qPCR, revealed an upregulation of Jak-2 and a compromised expression of several members within the Jak/Stat signaling pathway which could not be detected in either *db/db* C57BLKS or diabetic STZ-treated DBA/2J mice [50].

More recently, Woroniecka et al. performed the transcriptome analysis on microdissected kidney biopsies from DN patients, healthy living transplant donors, and patients undergoing tumor nephrectomies (analyzing the histologically normal kidney tissue). The microarray-derived expression profiles indicated that several podocyte-specific transcripts were downregulated, including PLCE1, PTGDS, NPHS1, NPHS2, SYNPO, PLA2R1, WT1, CLIC5, and PODXL. Glomerular transcripts showing upregulation included IGH, C3, COL1A2, CXCL6, and COL6A3. In the tubular compartment instead, authors detected increased expression of different transcripts including IGH, IGL, COL1A2, and COL3A1 [37].

Several reports analyzed the gene expression of both the glomerular and tubular compartments of T2DN kidney biopsies. Among the mRNA transcripts detected as enriched in the glomerular compartment of T2DN individuals are MRP8 [52], WNT1, WNT2B, WNT4, WNT6, WNT16, DKK3, and Lef1 [42], PKCα [58], FSP1 [44], ANGPTL2 [30], and ACE [26]. Decreased expression for ACE2 [26], VEGF [64, 106], CTGF, nephrin, podocin, and WT1 [41] was also reported in T2DN glomeruli.

When assaying microdissected, tubule-rich renal biopsies from patients with T2DN, IHG-1 [48], IL6, CCL2 CD68, and CCR5 [38] were increased, while TLR4 was overexpressed in both glomeruli and tubules of microalbuminuric and overt DN [38].

Using biopsy material collected by the European Renal cDNA Bank, the gene expression of tubulointerstitial mRNA from human DN kidneys was compared to that of living donors, cadaveric donors, and patients with minimal change disease through a combined microarray profiling and qPCR validation approach. Results indicated dysregulation of specific NF-κB targets, highlighting the existence of an inflammatory signature characteristic of progressive DN. Eight genes in particular were induced in T1DN and T2DN relative to controls: CCL5/RANTES, CXCL10/IP10, EDN1, VCAM1, HLA-A, HLA-B, IFNB1, and B2M [35].

Further work performed using the European Renal cDNA Bank material highlighted additional mRNA transcripts as dysregulated in T2DN kidney when compared to normal tissue. Within the glomerular compartment in particular, NRP1 and NRP2 were significantly lower in T2DN [55], while SMPDL3b was increased [61]. Within the tubulointerstitial compartment, upregulation of MMP7 [51] and FGF-2 [43], of the unfolded protein response genes HSPA5, HYOU1, and XBP1 [47] and of the apoptosis-related genes TRAIL and OPG [56], were observed.

In other cases, the expression of several transcripts was assessed on whole T2DN kidney tissue.

Upregulated mRNAs included HDAC2, HDAC4, and HDAC5 [34], B7-1 [36], Stat1 [33], TNFAIP8 and TIPE2 [62], PRKC-beta [59], VEGF [65], UII and UT [63], PDGF-A and PDGF-B [57], LOX1, LDLR, and CD36 [24], Jagged1/Hes1 [46], and Gremlin [45, 46].

Decreased transcription was detected for autophagy-related genes Beclin 1, LC3 [33, 34] and ATG7 [34], CXCL16, ABCA1, ABCG1, and apoE [24], Timp3, FoxO1 and FoxO3A, Atg5, and Atg8 [33], ANKRD56 and ENTPD8 [31], and nephrin [54].

In other works the study design was developed to compare T2DN with other glomerulopathies. Using a qPCR based approach, the tubulointerstitial compartment isolated from kidney biopsies of both DN patients, living donors, and minimal change disease patients was profiled specifically for the expression of 202 candidate genes involved in molecular pathways contributing to DN progression. Results showed a decreased expression of VEGF and EGF, while Collagens I and IV, fibronectin 1, and vimentin as well as matrix metalloproteinases 2, 7, and 14 and tissue inhibitor of metalloproteinases 1 and 3 were increased [39]. In another study, increased IRS2 mRNA was detected in DN patients compared to controls, while no significant changes IRS2 expression were present in biopsies from patients with focal-segmental glomerulosclerosis or membranous nephropathy [49].

Low expression of ROBO2 mRNA was present in DN compared to nephrosclerosis, focal-segmental glomerulosclerosis, membranous nephropathy, and control pretransplant biopsies [60].

A strong specific induction of COL8A1 and COL8A2 mRNAs expression was found in both glomerular and tubular compartments of biopsies from patients with T2DN versus control pretransplant biopsies, benign nephrosclerosis, and focal-segmental glomerulosclerosis [40]. Finally, increased ACE expression was observed in T2DN biopsies compared to benign nephrosclerosis, minimal change nephrotic syndrome, and lupus nephritis [25].

Aiming to develop a diagnostic tool for early DN diagnosis, Zheng et al. designed a PCR-array platform to detect expression changes in 88 genes simultaneously and employed it in a pilot study where the urinary sediment of DN patients was assayed. Authors found that several mRNAs were significantly increased in DN compared to healthy controls, in particular, NOTCH3, ACTN4, CDH2, ACE, FAT1, COL4A1, SYNPO, and TWIST1 [27]. Similar studies investigated the mRNA derived from the urinary sediment of T2DN patients. Increased mRNA levels of podocalyxin, CD2-AP [29], nephrin, WT-1 [28], α-actinin 4 podocin, and synaptopodin [28, 29] were found in the DN group compared with controls. Finally, in another work, authors claim that urinary expression of nephrin and podocin was useful for distinguishing diagnostic groups (IgA nephropathy, minimal change disease, and membranous nephropathy) as well as predicting renal function decline [53].

3.2. Noncoding RNA Studies. Until a few years ago, the molecular profiling of DN was mainly focused on the characterization of mRNA transcripts. Over the last decade however, much interest has converged toward the profiling of noncoding RNA (ncRNA) molecules. The ability of ncRNAs to modulate gene expression along with the discovery that they can be detected in biofluids and are fairly stable makes them ideal biomarker candidates.

microRNAs (miRNAs) are probably the most studied ncRNAs; they are short, single-stranded, highly conserved, and tissue-specific. miRNAs regulate protein synthesis through perfect partial match binding to their precursor messenger RNA. The partial match binding feature allows miRNAs to bind hundreds of targets simultaneously; accordingly the dysregulation of even one single miRNA molecule can profoundly influence the gene expression profile of the surrounding environment. For a complete review on miRNAs biogenesis and function refer to [107, 108]. In the field of DN, the majority of miRNA's profiling studies was performed on cellular and animal models. More recently, with the surprising discovery that miRNAs can be released and carried into the extracellular environment, different body fluids are being characterized in their miRNA's content.

The first miRNA to be recognized as relevant contributor to DN progression was miR-192 [109]. Initially identified in a mice model of DN, miR-192, along with miR-377, miR-337, and miR-129, was later discovered as being enriched in human mesangial cells (MCs) exposed to high glucose [67]. Interestingly, when assessing miR-192 in human DN kidney, expression levels not only are reduced but also inversely correlate with severity of kidney disease [72], raising once again the issue about the appropriateness of the currently available animal models for DN.

miR-21 has recently emerged as a marker for fibrosis in many complications [110, 111]; unsurprisingly, increased miR-21 expression was also detected in human T2DN kidney biopsies relative to healthy controls [74].

Except for the previously mentioned DN kidney profiling from Krupa et al., the array-based miRNome analysis of T2DN kidneys was recently published by Huang et al. and uncovered miR-155 and miR-146a enrichment in these samples [69]. These two are the only works describing the miRNome of human DN kidney; noteworthy, the existence of strict renal biopsy policies in most nephrology clinics might be a limiting factor in terms of sample collection and availability. In parallel, the urgent need for novel biomarkers of diagnosis and progression shifted priority to the profiling of more accessible samples, such as biological fluids.

Using a qPCR based approach, Argyropoulos et al. were the first to perform the urinary miRNA profiling of T1D patients with and without proteinuria. Results showed that miR-323b-5p, miR-221-3p, miR-524-5p, and miR-188-3p were underexpressed in albuminuric relative to nonalbuminuric patients, while miR-214-3p, miR-92b-5p, hsa-miR-765, hsa-miR-429, miR-373-5p, miR-1913, and miR-638 were overexpressed [71]. In a similar study performed on the RNA content of urinary exosomes, authors showed that miR-130a and miR-145 were enriched in T1D patients with microalbuminuria compared to normoalbuminuric subjects, while miR-155 and miR-424 were reduced [68].

In a work aimed to determine the urinary levels of all miR-29 family members (miR-29a, miR-29b, and miR-29c), miR-29a was significantly increased in albuminuric T2DN patients compared to normoalbuminuric patients and it also correlated with the degree of albuminuria [75].

In the work from Szeto et al., when comparing the urinary sediment of patients with either IgA nephropathy, DN, or hypertensive nephrosclerosis, miR-15 was decreased in DN samples compared to other groups [70]. Similarly, in another work authors found that miR-192 levels were reduced in urinary sediment of DN patients compared to both healthy controls and patients with either minimal change nephropathy, focal glomerulosclerosis, membranous nephropathy, or other diagnosis groups [73].

miRNAs expression was also measured in venous blood from T2D Han Chinese patients with and without albuminuria. Using a microarray-based approach, authors identified several differentially expressed miRNAs in the different study population and confirmed miRNA let-7a downregulation using qPCR. Very interestingly, authors also observed how the distribution of a specific variant within let-7a (rs1143770) was significantly higher in diabetic patients (with and without albuminuria) relative to control subjects [76].

Finally, dysregulation of a new class of noncoding RNA molecules has emerged as being potentially involved in different complications, including kidney disease. Among these noncoding RNA molecules, recent effort aims to characterize the so-called long noncoding RNAs (lncRNAs). Compared to miRNAs lncRNAs are longer than 200 nucleotides and are poorly conserved. This led to the initial assumption that lncRNAs were not biologically relevant. Today we know that lncRNAs contain individual domains and structural motifs that allow them to specifically associate with DNA, RNA, and/or protein and thus regulate their function.

The first lncRNA identified in kidney disease was PVT1. As previously discussed, multiple experimental evidence, from different ethnic populations, suggested a link between diabetic kidney disease and genetic variants within the PVT1 locus [22, 23]. PVT1, whose increase is significant in

Table 4: Epigenetic markers. List of epigenetic marks identified in DN. List is ordered alphabetically.

Locus	Sample type	Type of modification	Diabetes type	Potential value of biomarker	References
SHC1	PBMC	Reduced promoter methylation	—	Diagnostic	[77]
UNC13B	Whole blood	Increased DNA methylation	T1D	Diagnostic/prognostic	[78]

mesangial cells stimulated with high glucose, can induce the expression of plasminogen activator inhibitor 1 (PAI-1) and transforming growth factor beta 1 (TGF-β1) [112]. Noteworthy, six different miRNAs are encoded within the PVT1 gene; therefore, authors investigated whether an alteration in PAI-1 and TGF-β1 gene expression was ascribable to the PVT1 lncRNA transcript itself or whether it was the result of a mutation within the miRNAs encoded in the PVT1 gene. Results showed that both PVT1 lncRNA and miR-1207-5p were induced by high glucose independently and they both contributed to ECM accumulation in the kidney [66].

4. Epigenetic Studies in DN

The term epigenetics refers to all those dynamic structural changes that, while not resulting from an alteration in the DNA sequence, affect gene expression and can be inherited. Epigenetic modifications, such as DNA methylation, histone methylation, and histone acetylation, modify the accessibility of the chromatin and thus modulate transcription. They are responsible for the phenotypic differences within cell types and explain why the gene expression profile of an organism can change so profoundly during development. Unlike genetics, epigenetics is highly susceptible to influences from the environment; therefore, the understanding of its regulatory machinery offers an incredible opportunity for disease management.

The study of epigenetics in diabetic kidney disease is still in its embryonic phase although increasing evidence indicates metabolic memory as a consequence of long-lasting epigenetic modifications contributing to DN progression [113]. In 2007 Geisel et al. analyzed the promoter methylation of the stress response protein p66Shc, previously shown to increase susceptibility to oxidative stress and atherosclerosis [114]. In peripheral blood mononuclear cells isolated from ESRD patients and control subjects, authors demonstrated that increased p66Shc expression in ESRD group was linked to a significant reduction in the methylation of its promoter region [77].

Using an array based approach, the genome-wide promoter DNA methylation of 192 T1D patients was analyzed searching for any possible association with DN. The analysis was conducted using DNA extracted from peripheral blood cells as these include the T cell population responsible for islet beta cells destruction in T1D. Importantly, among the several CpG islands showing correlation with DN development, results uncovered one in particular (rs10081672), located upstream of the UNC13B gene. Additionally, this region is in strong linkage disequilibrium with rs13293564, a variant associated with DN susceptibility. Importantly, depending on which allele is present in rs10081672, a CpG site is either created or abrogated, thereby affecting transcription factor binding [78].

In another work, the genome-wide DNA methylation of diabetic patients with ESRD and diabetic patients without nephropathy was compared with the aim to identify novel disease biomarkers for noninvasive diagnosis. Patients' saliva was employed as starting material for DNA extraction while the study population included African Americans and Hispanic individuals. Results highlighted differential methylation at two or more CpG sites in 187 genes between the two groups. Interestingly, many of these genes are involved in inflammation, oxidative stress, ubiquitination, fibrosis, and drug metabolism, and some in particular are even known for their genetic association with DN, suggesting once again a very close connection between genetic dysregulation and epigenetic dysregulation in the pathogenesis of DN [115].

A recent paper from Hasegawa et al. demonstrated that Sirt1, a protein deacetylase that targets histones and transcription factors, is reduced in STZ-treated mice. Using a transgenic mouse model authors also elucidated the interaction between Sirt1 expression and CpG methylation of Cldn1, a gene encoding for the protein Claudin-1. Claudin-1 is a tight junction protein involved in cell-to-cell adhesion and authors suggest that its epigenetic-mediated induction is responsible for podocyte effacement and proteinuria. In support of this hypothesis authors also revealed the correlation between proteinuria and Sirt1 expression in human DN kidney [116].

Finally, Reddy et al. elegantly demonstrated the link between the protective effect of angiotensin II receptor antagonist, losartan, and its ability to reverse specific epigenetic modifications in the glomeruli of diabetic *db/db* mice [117]. The epigenetic marks cited in this paper are listed in Table 4.

All these experimental evidences show that epigenetics holds the potential to allow a temporary and reversible manipulation of the gene expression, conferring protection from disease progression. They also highlight the importance of understanding the epigenetic contribution to DN progression.

5. Proteomics Studies in DN

The proteome probably represents the most complete expression of the potentialities of a living organism since it focuses on the set of proteins, expressed by the genome, that regulate biological and metabolic cell function. The "proteomics," formally defined as the massive and mass spectrometric-based analysis of the proteome, is a complex and interdisciplinary matter requiring expertise spanning from chemistry to biology and bioinformatics, in order to reveal the meaning of complex protein datasets of a biological sample in physiological and pathological conditions. Unlike

genomics studies, based on the analysis of biological samples that may be expanded artificially making complex studies from little starting material possible, proteomics requires a larger amount of starting sample that can be easily available in biological fluids rather than in the tissues or cells. For this reason, proteomic studies in nephrology are more oriented to the analysis of biological fluids and have led, in the last decade, to the identification of a number of putative biomarkers that are expected to enter shortly into the clinical practice [118].

In the next paragraphs we will discuss the main application of proteomics to the identification of new potential biomarkers of DN in kidney tissues and biological fluids with a special emphasis on the new emerging potentialities of the post-translational modifications (PTMs) screenings. The proteomic markers discussed in this paper are also reported in Table 5.

5.1. Kidney Tissue. Glomerular damage plays a critical role in the onset of DN making this renal compartment a key target for proteomic investigation [119]. However, only few proteomic studies have been carried out on isolated glomeruli since, in general, renal biopsy is rarely carried out on diabetics patients and the number of isolated glomeruli, when starting form biopsy material, is too scarce to produce homogeneous preparations of individual specimens and to extract adequate glomerular protein amounts for deep proteomic studies. Recent methodological improvements [120] have now permitted the extraction of intact and unmodified proteins from formalin fixed paraffin embedded (FFPE) samples thus making available the use of vast archive of kidney tissues for proteomic analysis. Proteomic analysis of isolated glomeruli, obtained by Laser Capture Microdissection (LCM) [121], allowed the identification of over 100 differentially expressed tissue proteins between DN and nondiabetic glomeruli [79]. Notably, the results of this study probably underestimates the differences of the glomerular proteome since it was carried out on FFPE tissues derived from autopsy cases undergoing postmortem proteolysis [122]. However, among differently expressed proteins, nephronectin, a protein implicated in the assembly of extracellular matrix and nephrogenesis [123], was confirmed as differently expressed in DN tissue specimens using immunohistochemistry. A similar study reported increased expression of C3 and the membrane attack complex (C5b-9) and a marked reduction of podocyte-associated proteins and antioxidant proteins in DN [80]. Even if these proof of concept studies demonstrate the usefulness of FFPE tissue proteomics, the potentialities of this approach are still prevented by the poor availability of tissue specimens that limits the identification of the key molecular events involved in the onset and progression of DN.

5.2. Biofluids. Biofluids encompass any liquid originating from inside the bodies of living organism. Among the body fluids proteomics has been mostly applied to urine and serum/plasma. Rossing and colleagues reported, in urine of T1D patients with DN, a panel of 65 urine biomarkers, mainly composed of collagen fragments, that was further validated in a multicentre independent cohort of T2DM patients [124, 125]. Zürbig et al. expanded the 65 peptides classifier to 273 and demonstrated its ability to predict the occurrence of the microalbuminuria in T1D and T2DM normoalbuminuric patients [126, 127]. These data were recently confirmed in another independent study that specifically identified subsets of urine biomarkers able to predict to the transition from normo- to microalbuminuria or from micro- to macroalbuminuria [81] indicating that the appearance of collagen fragments in urine of T2DM patients may have both diagnostic and prognostic values. Potential predictive biomarkers have been also described in urine samples of T1 diabetic patients [82]. LC/MS/MS analysis of 22 T1D normoalbuminuric patients developing microalbuminuria after 6 years median follow-up allowed identifying a set of potential predictive biomarkers that were further validated by ELISA assay. Of note, the introduction of these proteomic biomarkers (THP, progranulin, alpha-1-glycoprotein, and clusterin) into the baseline model that included diabetes duration, baseline Albumin Excretion rate (AER), HbA1c, cystatin C, and uric acid improved the prediction of renal function worsening from 84% to 89%.

Jin et al. used *Isobaric Tags for Relative and Absolute Quantification* (iTRAQ) and LC/MS/MS to quantify and identify a set of urinary proteins differentially excreted between normoalbuminuric and microalbuminuric T2DM patients. Three protein biomarkers, namely, alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, and prostate stem cell antigen, were included in a multiplex assay that was able to correctly classify normoalbuminuric and microalbuminuric T2DM patients with about 92% accuracy [83].

Dihazi et al. identified and validated, by SELDI-TOF/MS, two mass peaks corresponding to B2-microglobulin and ubiquitin ribosomal fusion protein that were selectively and differently excreted in nephropathic diabetic patients [84]. We further refined this study by selecting only diabetic patients with biopsy-proven Kimmelstiel-Wilson lesions and identifying both urinary B2-microglobulin and free ubiquitin as specific biomarkers of diabetic glomerulosclerosis over other nondiabetic kidney lesions [85]. Although the overall analysis of the urine proteome is up to now the most used way to search for disease-specific biomarkers, the future of this matter will be the analysis of well-purified proteins subfractions since it may provide more detailed information about simplified proteomes and potentially improve the knowledge of specific pathways. Until few years ago, the most useful way to reduce the proteome complexity was the selective antibody-based depletion of the most abundant proteins. In the last few years, the enrichment of post-translationally modified proteins has begun a new strategy to highlight functionally interesting proteins. Two emerging branches in this context are phosphoproteomics and glycoproteomics. Protein phosphorylation is a key player in the regulation of most cell pathways; thus, phosphoproteome screening of urine samples may represent a precious source of information about deregulated cell processes in many kidney diseases including DN. However, up to now, urine phosphoproteome analysis has not been applied yet to soluble proteins in DN and other CKD probably because most of the historical collections of urine samples have not been prepared and

Table 5: Proteomic biomarkers. List of significant protein biomarkers ordered as they are cited in the text. LCM: Laser Capture Microdissection; LC/MS/MS: liquid chromatography coupled to tandem mass spectrometry; IF: immunofluorescence; IHC: immunohistochemistry; SELDI-TOF/MS: Surface Enhanced Laser Desorption Ionization Mass Spectrometry; CE-MS: capillary electrophoresis; 2DE: two-dimensional electrophoresis.

Protein	Code	Sample	Expression	Assay type	Diabetes type	Potential value of biomarker	References
Integrin, alpha 1	ITGA1	FFPE Kidney	Up	LCM + LC/MS/MS; IHC	T2D	Diagnostic	[79]
Laminin, beta 2	LAMB2, LAMS	FFPE Kidney	Up	LCM + LC/MS/MS; IHC	T2D	Diagnostic	[79]
Nephronectin	NPNT, EGFL6L, POEM, and UNQ295/PRO334	FFPE Kidney	Up	LCM + LC/MS/MS; IHC	T2D	Diagnostic	[79]
Actinin, alpha 4	ACTN4	FFPE Kidney	Up	LCM + LC/MS/MS; IHC	T2D	Diagnostic	[79]
C3	C3, CPAMD1	FFPE Kidney	Up	LCM + LC/MS/MS; IF	T2D	Diagnostic	[80]
C5b-9	C5, CPAMD4	FFPE Kidney	Up	LCM + LC/MS/MS; IF	T2D	Diagnostic	[80]
Fibrinogen α-chain	FGA	FFPE Kidney	Up	LCM + LC/MS/MS; IF	T2D	Diagnostic	[80]
Synaptopodin	SYNPO, KIAA1029	FFPE Kidney	Up	LCM + LC/MS/MS; IF	T2D	Diagnostic	[80]
Collagen α-1 (I) chain	CO1A1_HUMAN	Urine	Down	CE-MS	T2D	Prognostic	[81]
Collagen α-1 (III) chain	CO3A1_HUMAN	Urine	Down	CE-MS	T2D	Prognostic	[81]
Collagen α-2 (I) chain	CO1A2_HUMAN	Urine	Down	CE-MS	T2D	Prognostic	[81]
Neurosecretory protein VGF	VGF_HUMAN	Urine	Down	CE-MS	T2D	Prognostic	[81]
Osteopontin	OSTP_HUMAN	Urine	Down	CE-MS	T2D	Prognostic	[81]
Polymeric immunoglobulin receptor	PIGR_HUMAN	Urine	Down	CE-MS	T2D	Prognostic	[81]
Serum albumin	ALBU_HUMAN	Urine	Up	CE-MS	T2D	Prognostic	[81]
Sodium/potassium-transporting ATPase γ chain	ATNG_HUMAN	Urine	Down	CE-MS	T2D	Prognostic	[81]
Pro-SAAS	PCSK1_HUMAN	Urine	Up	CE-MS	T2D	Prognostic	[81]
α-2-HS-glycoprotein	FETUA_HUMAN	Urine	Up	CE-MS	T2D	Prognostic	[81]
a-1 acid glycoprotein	AGP	Urine	Up	SDS-PAGE + LC/MS/MS + ELISA	T1D	Prognostic	[82]
Clusterin	CLU, APOJ, CLI, KUB1, and AAG4	Urine	Comparable	SDS-PAGE + LC/MS/MS + ELISA	T1D	Prognostic	[82]
Progranulin	GRN	Urine	Up	SDS-PAGE + LC/MS/MS + ELISA	T1D	Prognostic	[82]
Tamms-Horsfall glycoprotein	THP	Urine	Up	SDS-PAGE + LC/MS/MS + ELISA	T1D	Prognostic	[82]
Alpha-1-acid glycoprotein 1	ORM1, AGP1	Urine	Up	iTRAQ labelling + LC-MS/MS; WB	T2D	Diagnostic	[83]

Table 5: Continued.

Protein	Code	Sample	Expression	Assay type	Diabetes type	Potential value of biomarker	References
Alpha-1-antitrypsin	SERPINA1, AAT, PI, PRO0684, and PRO2209	Urine	Up	iTRAQ labelling + LC-MS/MS; WB	T2D	Diagnostic	[83]
Prostate stem cell antigen	PSCA, UNQ206/PRO232	Urine	Up	iTRAQ labelling + LC-MS/MS; WB	T2D	Diagnostic	[83]
Ubiquitin ribosomal fusion protein (UbA52)	UBA52, UBCEP2	Urine	Up	SELDI-TOF/MS, WB	T2D	Diagnostic	[84]
β2-microglobulin	B2M, CDABP0092, and HDCMA22P	Urine	Up	SELDI-TOF/MS, WB, ELISA	T2D	Diagnostic	[84, 85]
Free ubiquitin	UBB, UBC, UBA52, and RPS27A	Urine	Up	SELDI-TOF/MS, WB, ELISA	T2D	Diagnostic	[85]
Histone-lysine N-methyltransferase 2C	KMT2C, HALR, KIAA1506, and MLL3	Urine exosomes	Up	2DE + LC/MS/MS	T2D	Diagnostic	[86]
Voltage-dependent anion-selective channel protein 1	VDAC1, VDAC	Urine exosomes	Down	2DE + LC/MS/MS	T2D	Diagnostic	[86]
Alpha-1-microglobulin/ bikunin precursor	AMBP, HCP, and ITIL	Urine exosomes	Up	2DE + LC/MS/MS	T2D	Diagnostic	[86]
Vasorin (glycated)	VASN, SLITL2, UNQ314/PRO357/ PRO1282	Plasma	Up	LC/MS/MS; WB	T2D	Diagnostic	[87]
Retinol binding protein-4 (glycated)	RBP4, and PRO2222	Plasma	Up	LC/MS/MS; WB	T2D	Diagnostic	[87]
Lumican (glycated)	LUM, LDC, SLRR2D	Plasma	Up	LC/MS/MS; WB	T2D	Diagnostic	[87]
Vasorin (glycated)	VASN, SLITL2, and UNQ314/PRO357/ PRO1282	Plasma	Up	LC/MS/MS; WB	T2D	Diagnostic	[87]
Hemopexin precursor (glycated)	MMP15	Plasma	Up	2DE; ESI-Q-TOF/MS/MS	T2D	Diagnostic	[88]
Alpha-1-antitrypsin (glycated)	SERPINA1, AAT, PI, PRO0684, and PRO2209	Plasma	Up	2DE; ESI-Q-TOF/MS/MS	T2D	Diagnostic	[88]
Haptoglobin-related protein (glycated)	HPR	Plasma	Up	2DE; ESI-Q-TOF/MS/MS	T2D	Diagnostic	[88]
Serine proteinase inhibitor (glycated)	SERPINA5, PCI, PLANH3, and PROCI	Plasma	Up	2DE; ESI-Q-TOF/MS/MS	T2D	Diagnostic	[88]
Complement factor C4B3 (glycated)	C4B, CO4, CPAMD3, and C4B_2	Plasma	Up	2DE; ESI-Q-TOF/MS/MS	T2D	Diagnostic	[88]
Prekallikrein (glycated)	KLKB1, KLK3	Plasma	Up	2DE; ESI-Q-TOF/MS/MS	T2D	Diagnostic	[88]
Apolipoprotein (ApoE)	APOE	Plasma	Up	2DE; MALDI-TOF/MS/MS	T2D	Diagnostic	[89]
Glutathione peroxidase (eGPx)	GPX2	Plasma	Up	2DE; MALDI-TOF/MS/MS	T2D	Diagnostic	[89]
Vitamin D-binding protein (DBP)	GC	Plasma	Up	2DE; MALDI-TOF/MS/MS	T2D	Diagnostic	[89]

stored in presence of phosphatases inhibitors that, preventing the liability of this PTMs, may ensure more reproducible results. On the contrary, the analysis of the microvesicular fraction (i.e., exosomes) that originates from renal epithelial cells and are released into urine may be, at the moment, more useful to study this kind of PTM as the presence of the exosomes' membrane may preserve PTMs by protecting their protein content from spontaneous degradation and dephosphorylation by proteases or phosphatases, respectively [128]. Zubiri et al. have already published the first proteomic study on urine exosomes of DN patients demonstrating the potentiality of this microvesicular screening for identifying DN specific biomarkers [86]. Specifically, 3 over the 25 most significant differently expressed proteins, namely, voltage-dependent anion-selective channel protein 1 (VDAC1), Isoform 1 of histone-lysine N-methyltransferase MLL3, and alpha-1-microglobulin/bikunin precursor (AMB), were also validated. Of note, MLL3, a specific tag for epigenetic transcriptional activation, was detected only in DN exosomes, thus emphasizing the potential importance of epigenetic mechanisms in the pathophysiology of DN. Furthermore, Gonzales et al. have recently reported the first phosphoproteomic screening of the urine exosomes in healthy subjects [129]. It is reasonable to think about the forthcoming application of the exosomes' phosphoproteomics as a new way to identify specific deregulated patterns in kidney diseases. As for phosphoproteomics also glycoproteomics of urine samples is in its infancy. At the moment, only one paper has applied this approach to the study of CKD identifying a number of urinary proteins involved in immune/stress response and many biological functions like homeostasis, platelet degranulation and coagulation, transport, and secretion [130]. Due to the importance of the glycoproteomics in cell-cell interaction and signalling cascades, it is reasonable that many further studies will be planned in the next year to understand, by screening this specific subset of proteins, the molecular mechanisms involved in damage progression of specific nephropathies including DN. Interestingly, the usefulness of the glycoproteomics for the diagnosis of DN has been recently reported in plasma where thirteen significantly upregulated glycoproteins were described in DN patients compared to T2DM patients without nephropathy [87]. Among these, increased plasma levels of glycated lumican, vasorin, and retinol binding protein-4 were validated by immunoblotting and showed potential specificity for DN. By using a different proteomic strategy, Kim and coworkers reported that increased plasma levels of glycated PEDF, apolipoprotein J precursor, hemopexin, immunoglobulin mu heavy chain, and immunoglobulin kappa chain correlated with poor glycaemic control in T2DM patients while glycated prekallikrein and complement factor C4B3 correlated with microalbuminuria and other glycated proteins such as hemopexin precursor, serine proteinase inhibitor, alpha-1-antitrypsin, and haptoglobin-related protein were associated with DN [88]. These studies confirmed the potentiality of the plasma glycoproteome for the identification of reliable biomarkers of DN and their importance is emphasized by the consideration that the overall analysis of serum/plasma proteome is challenging because the candidate biomarkers are generally present in trace amounts. Of note, there is an alternative way to reduce the complexity of this biological fluid, namely, the prefractionation of the samples, achieved by several known strategies before the analysis [131], that allow removing the large background of nonrelevant and abundant proteins and may favour the discovery of potential candidate biomarkers. Up to now only few studies have used this approach to analyse the serum [89, 132] or plasma [133] proteome of T2DM patients. These studies have reported extracellular glutathione peroxidase (eGPx) and apolipoprotein (ApoE) as potential diagnostic biomarkers of DN and vitamin D-binding protein (DBP) as early biomarker of renal damage in T2DM. Overall many independent studies are showing an increasing number of new biomarkers that are potentially useful for both early diagnosis and monitoring of the disease and to understand ever more deeply its pathogenesis.

6. Metabolomics Studies on DN

Metabolomics is a systematic evaluation of small molecules (i.e., metabolites) that may provide fundamental biochemical insights into disease pathways, drug toxicity, and gene function. Metabolomics profiling is generally carried out by Nuclear Magnetic Resonance (NMR) and MS-based profiling each with advantage and limitations [134]. Two main strategies may be adopted for metabolomics analysis of biological samples: targeted and untargeted profiling. The targeted profiling focuses only on sets of few metabolites generally included in specific metabolic pathways while untargeted analysis provides a comprehensive evaluation of the metabolome without any *a priori* hypothesis on the metabolic pathways. Targeted analysis is an essential tool for the investigation of biological mechanisms rather than for biomarkers discovery; in fact it is a quantitative approach that allows quantification of each metabolite of an interested metabolic pathway through the use of isotope-labelled standards [135]. Untargeted approach is instead more suitable for biomarker discovery since the whole metabolic profile of cases and controls may allow identification of disease-correlated biomarkers. As obvious, the latter approach needs, as for proteomics, further data analysis through supervised statistical methods in order to construct disease-specific metabolomics classifier further sequenced by mass spectrometry. In the last years, the optimization of the separation techniques has allowed the selectively purification of specific class of metabolites such as phospholipids and fatty acids, leading to the development of new more focused untargeted analysis such as "phospholipidomics." As for proteomics, most of the metabolomics studies have been carried out on biofluids, namely, urine and serum/plasma. All metabolomic markers cited in this paper are also reported in Table 6.

6.1. Urine Metabolomics. Urine metabolomics may offer direct insights into biochemical pathways linked to kidney dysfunction since a variety of metabolites are concentrated by the kidney and excreted in urine. Sharma et al. [90] used targeted analysis to investigate the urinary excretion of 94 metabolites in healthy subjects (HS) and T2DM patients with (DM+CKD) or without (DM-CKD) CKD. Thirteen

Table 6: Metabolomics biomarkers. List of significant metabolites ordered as they are cited in the text. LC/MS/MS: liquid chromatography coupled to tandem mass spectrometry; GC-MS: gas chromatography/mass spectrometry; NMR: Nuclear Magnetic Resonance; CE-MS: capillary electrophoresis; HPLC–UV/MS/MS: high pressure liquid chromatography coupled to UV and mass spectrometry; UPLC–oa-TOF-MS: ultra performance liquid chromatography coupled to time of flight mass spectrometry.

Metabolite	Code	Sample	Expression	Assay type	Diabetes type	Potential value of biomarker	References
2-Methyl acetoacetate	HMDB03771	Urine	Down	GC-MS	T2D	Diagnostic	[90]
3-Methyl adipic acid	HMDB00555	Urine	Down	GC-MS	T2D	Diagnostic	[90]
3-Methyl crotonyl glycine	HMDB00459	Urine	Down	GC-MS	T2D	Diagnostic	[90]
2-Ethyl 3-OH propionate	HMDB00396	Urine	Down	GC-MS	T2D	Diagnostic	[90]
3-Hydroxyisobutyrate	HMDB00435	Urine	Down	GC-MS	T2D	Diagnostic	[90]
3-Hydroxyisovalerate	HMDB00754	Urine	Down	GC-MS	T2D	Diagnostic	[90]
3-Hydroxypropionate	HMDB00700	Urine	Down	GC-MS	T2D	Diagnostic	[90]
Aconitic acid	HMDB00958	Urine	Down	GC-MS	T2D	Diagnostic	[90]
Citric acid	HMDB00094	Urine	Down	GC-MS	T2D	Diagnostic	[90]
Glycolic acid	HMDB00115	Urine	Down	GC-MS	T2D	Diagnostic	[90]
Homovanillic acid	HMDB00118	Urine	Down	GC-MS	T2D	Diagnostic	[90]
Tiglylglycine	HMDB00959	Urine	Down	GC-MS	T2D	Diagnostic	[90]
Uracil	HMDB00300	Urine	Down	GC-MS	T2D	Diagnostic	[90]
Butenoylcarnitine	HMDB13126	Plasma	Up	GC-MS	T2D	Prognostic	[91]
Glutamine	HMDB00641	Urine	Down	GC-MS	T2D	Prognostic	[91]
Hexose	HMDB33704	Urine	Down	GC-MS	T2D	Prognostic	[91]
Histidine	HMDB00177	Plasma	Down	GC-MS	T2D	Prognostic	[91]
Tyrosine	HMDB00158	Urine	Down	GC-MS	T2D	Prognostic	[91]
Hippuric acid	HMDB00714	Urine	Down	LC-MS	T1D	Prognostic	[92]
S-(3-Oxododecanoyl) cysteamine	HMDB59773	Urine	Up	LC-MS	T1D	Prognostic	[92]
Substituted carnitine	HMDB00062	Urine	Up	LC-MS	T1D	Prognostic	[92]
3-OH-isovalerate	HMDB00754	Serum	Down	NMR	T2D	Diagnostic	[93]
4-Aminobutyrate + (CH-CH2-CH2-)	HMDB00112	Serum	Up	NMR	T2D	Diagnostic	[93]
Alanine	HMDB00161	Serum	Down	NMR	T2D	Diagnostic	[93]
Cholesterol	HMDB00067	Serum	Down	NMR	T2D	Diagnostic	[93]
Choline	HMDB00097	Serum	Down	NMR	T2D	Diagnostic	[93]
Creatine	HMDB00064	Serum	Down	NMR	T2D	Diagnostic	[93]
Creatine-P	HMDB01511	Serum	Down	NMR	T2D	Diagnostic	[93]
Creatinine	HMDB00562	Serum	Up	NMR	T2D	Diagnostic	[93]
Dimethylamine	HMDB00087	Serum	Down	NMR	T2D	Diagnostic	[93]
Glucose	HMDB00122	Serum	Up	NMR	T2D	Diagnostic	[93]
Glutamine	HMDB00641	Serum	Down	NMR	T2D	Diagnostic	[93]
Isoleucine	HMDB00172	Serum	Down	NMR	T2D	Diagnostic	[93]
Isoleucine	HMDB00172	Serum	Down	NMR	T2D	Diagnostic	[93]
Lactate	HMDB00190	Serum	Up	NMR	T2D	Diagnostic	[93]
Leucine	HMDB00687	Serum	Up	NMR	T2D	Diagnostic	[93]
Leucine + isoleucine	HMDB28932	Serum	Down	NMR	T2D	Diagnostic	[93]
Lipid (-CH3)	—	Serum	Up	NMR	T2D	Diagnostic	[93]
Lipids (beta-CH2)	—	Serum	Up	NMR	T2D	Diagnostic	[93]
Lipids (CH2-)n	—	Serum	Up	NMR	T2D	Diagnostic	[93]
N-Acetylglutamine	HMDB06029	Serum	Down	NMR	T2D	Diagnostic	[93]

Table 6: Continued.

Metabolite	Code	Sample	Expression	Assay type	Diabetes type	Potential value of biomarker	References
O-Phosphocholine	HMDB01565	Serum	Down	NMR	T2D	Diagnostic	[93]
Proline	HMDB00162	Serum	Down	NMR	T2D	Diagnostic	[93]
Pyruvate	HMDB00243	Serum	Down	NMR	T2D	Diagnostic	[93]
Trimethylamine	HMDB00906	Serum	Down	NMR	T2D	Diagnostic	[93]
Valine	HMDB00883	Serum	Down	NMR	T2D	Diagnostic	[93]
Valine + isoleucine	HMDB29130	Serum	Down	NMR	T2D	Diagnostic	[93]
Aspartic acid	HMDB00191	Serum	Up	CE-MS	T2D	Diagnostic	[94]
Azelaic acid	HMDB00784	Serum	Down	CE-MS	T2D	Diagnostic	[94]
Galactaric acid	HMDB00639	Serum	Down	CE-MS	T2D	Diagnostic	[94]
Symmetric dimethylarginine (SDMA)	HMDB03334	Serum	Up	CE-MS	T2D	Diagnostic	[94]
Dihydrosphingosine	HMDB00269	Serum	Down	UPLC–oa-TOF-MS	T2D	Diagnostic	[95]
Leucine	HMDB00687	Serum	Down	UPLC–oa-TOF-MS	T2D	Diagnostic	[95]
Phytosphingosine	HMDB04610	Serum	Down	UPLC–oa-TOF-MS	T2D	Diagnostic	[95]
Adenosine	HMDB00050	Plasma	Up	HPLC–UV/MS/MS	T2D	Diagnostic	[96]
Creatinine	HMDB00562	Plasma	Up	HPLC–UV/MS/MS	T2D	Diagnostic	[96]
Inosine	HMDB00195	Plasma	Down	HPLC–UV/MS/MS	T2D	Diagnostic	[96]
Uric acid	HMDB00289	Plasma	Up	HPLC–UV/MS/MS	T2D	Diagnostic	[96]
Xanthine	HMDB00292	Plasma	Up	HPLC–UV/MS/MS	T2D	Diagnostic	[96]
Phosphatidylinositol	HMDB06953	Plasma	Down	LC/MS/MS	T2D	Diagnostic	[97]
Sphingomyelin	HMDB12089	Plasma	Up	LC/MS/MS	T2D	Diagnostic	[97]
Arachidonic acid	HMDB01043	Plasma	Up	CG-MS	T2D	Prognostic	[98]

metabolites differently excreted between T2DM patients and HS were also useful to differentiate DM+CKD from DM-CKD. Interestingly, 5 out 13 metabolites were differently excreted between DN and other CKD, thus being specifically associated with the diabetic kidney disease while 8/13 reflected metabolic changes shared by diabetic and nondiabetic CKD. Most of the less excreted metabolites in DN group were water soluble organic anions and functional analysis correlated them with impaired mitochondrial function in DN. Very recently, Pena and colleagues carried out an untargeted analysis of urine and plasma metabolome by GC-MS and reported the possible usefulness of a set of metabolites to predict the development of DN on top of the traditional renal risk markers, namely, baseline urinary albumin excretion and baseline estimated glomerular filtration rate [91]. In this prospective study, 24 normo- to microalbuminuria case/controls pairs and 21 micro- to macroalbuminuria case/controls pairs were enrolled. The metabolomic profiles of micro- to macroalbuminuria case/control pairs show significant differences while normo- to microalbuminuria pairs remained unchanged. Specifically they reported two plasma metabolites (butenoylcarnitine and histidine) and three urine metabolites (hexose, glutamine, and tyrosine) significantly differentially excreted in microalbuminuric patients prone to develop macroalbuminuria. The area under receiving operating characteristic (ROC) curve arising from the integration of these urine and plasma metabolites to a reference model based on baseline eGFR and urine albumin excretion passed form 84% to 99% correct prediction. Although these results appear impressive, as the authors suggest, they still need to be managed with care until a validation study on larger and independent cohorts will be set up. Some of the identified metabolites may have direct link with the pathopysiology of diabetes and its chronic complications since, for example, butenoylcarnitine plasma accumulation has been related to the excessive yet incomplete mitochondrial oxidation of fatty acids [136], possibly attributable to a lower mitochondrial number and reduced oxidation capacity in T2D tissues [137] while histidine, a modulator of inflammation and oxidative stress, may be correlated with impaired inflammation and oxidative stress in T2DM and CKD patients. It is worth noting that both studies stressed the importance of mitochondria dysregulation in the pathogenesis of DN. Urine metabolomics has been also applied to type 1 diabetic patients in order to identify predictive biomarkers of renal function worsening [92]. Metabolite profile of baseline 24 h urine samples of 52 type 1 diabetic patients (26 stable normoalbuminuric and 26 progressed toward microalbuminuria in 5.5 years' follow-up) was carried out by LC/MS and GC-MS. Multivariate logistic regression analysis of GC-MS and LC/MS dataset showed 65% and 75% predictive power after cross-validation, respectively. Twenty-one and 14 compounds

showed a significant contribution to the logistic regression model based on GC-MS and LC/MS dataset, respectively. Most of the identified GC-MS compounds were carboxylic compounds, acidic metabolites, and endogenous amino acids not showing a documented direct relation to DN while LC-MS dataset reveals specific compounds related to impaired fatty acids metabolism, detoxification system, and gut microbiome.

6.2. Serum and Plasma Metabolomics. Serum and plasma metabolomics has been carried out of both whole samples and specific subfractions. Marrachelli and coworkers [93] performed both genomic and metabolomic screening of over 1500 Caucasian T2DM patients, characterized the serum metabolome profile of the microalbuminuric patients by Nuclear Magnetic Resonance (NMR), and correlated it with specific genotypes, thus reporting a potential predictive value of the genotype on the onset of microalbuminuria in T2DM. Furthermore, Hirayama et al. [94] reported, in T2DM patients, 19 serum metabolites including creatinine, aspartic acid, γ-butyrobetaine, citrulline, symmetric dimethylarginine (SDMA), kynurenine, azelaic acid, and galactaric acid that were positively correlated with albuminuria and negatively with eGFR. Of note, some of the most significantly differently excreted metabolites were not identified. Multiple logistic regression, carried out on identified metabolites, recognized 4 features, namely, aspartic acid, azelaic acid, galactaric acid, and symmetric dimethylarginine (SDMA) as relevant for the model and allowed correct identification of DN patients with about 75% accuracy. Zhang et al. [95] carried out serum metabolomic profiling of 8 DN patients, 33 type 2 diabetes mellitus (T2DM) patients, and 25 healthy volunteers in order to investigate the presence of DN biomarkers. Importantly, they reported significant changes of leucine, dihydrosphingosine and phytoshpingosinewere specifically in the DN cohort, thus suggesting the perturbations of amino acid metabolism and phospholipid metabolism as key events in diabetic disease. Other authors have instead investigated specific subfractions of the metabolome, namely, compounds linked to purine and pyrimidine metabolism, phospholipids, and fatty acids.

Xia et al. [96] standardized an analytical method for analysis and quantification of purine and pyrimidine metabolites in DN patients and matched healthy controls. According to the well-established association of the purine and pyrimidine metabolic pathway with the development of the DN, they could assess that uric acid, xanthine, and adenosine were significantly increased in DN patients (especially in those at stage V according to Mogensen classification) while inosine is reduced probably as a result of the adenosine deaminase inhibition that catalyzes inosine formation from adenosine. Impaired lipid metabolism has been directly associated with T2DM and DN. Several phospholipids (PLs), significantly upregulated or downregulated in disease models, have been already recognized as potential biomarkers of T2DM or DN [138, 139]. Comprehensive and quantitative analysis of plasma PLs, such as phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, sphingomyelin, and lysophosphatidylcholine, may selectively distinguish T2DM from DN patients [97]. Targeted quantification of the phospholipids revealed proportional decrease of phosphatidylinositol and linear increase of sphingomyelin in DN patients. Although the molecular pathogenetic mechanisms leading to impaired metabolism of phospholipids are not clear, the authors suggest that reduced phosphatidylinositol may reflect increased sorbitol pathway activation in T2DM while increased sphingomyelin may depend on glucocorticoids-mediated sphingolipids metabolism.

Also plasma fatty acids (FAs) may have a direct impact on the occurrence and development of diabetes since their abnormal accumulation in parenchymal cells of multiple tissues, called lipotoxicity, has been suggested as a trigger of T2DM and its chronic complications [140]. Specific metabolomics screening of FAs, namely, lipidomics, may contribute to the understanding of this disease. Han and colleagues reported a standardized method based on Gas Chromatography-Mass Spectrometry (CG-MS) useful for the specific assessment of nonesterified and esterified fatty acids (NEFAs and EFAs, resp.) [98]. Lipidomics screening of 150 patients including diabetics with and without nephropathy showed high discrimination power on different stage of DN. Disease progression was specifically correlated with plasma levels of arachidonic acid that is involved in the anabolism of prostaglandins, thus suggesting a key role of the inflammatory processes in the progression of DN.

7. Conclusion

As genetic studies conducted so far are still inconclusive, it is difficult to envisage a common genetic basis for the development of DN. Quite possibly a number of environmental factors contribute significantly toward the evolution of the diabetic patient to this specific complication. However, there is no doubt that, from the earliest stages of the disease, many molecular changes, observed at the transcriptomics, proteomics, and metabolomics level, anticipate the onset of a clinical phenotype and may allow us to reconstruct in detail the pathogenetic basis of kidney damage in T2DM. Although new omics challenges such as the analysis of the protein post-translational modifications and of multiprotein complexes, mimicking what naturally happen in intracellular behavior, will further broaden our understanding of the DN pathogenesis, we are already able to identify the common thread that unites all the disparate molecular changes described in the literature by performing bioinformatic-based analysis of genes, transcripts, proteins, and metabolites described so far. We can envisage that the selection of specific omic biomarkers and clinical phenotypes might lead to a better stratification of patient's specific "type" of renal damage in T2DM and might allow the identification of patients that progress or respond to a specific therapy. To accomplish this task and go forward, however, there is an urgent need to build up disease-specific platforms containing personal, clinical, and omics profiles that will allow the full potential application of systems biology analysis and the development of specific disease phenotype models.

We can expect in the next future the development of new paradigms of renal damage in T2DM that will contribute to defining of the road to the molecular medicine as a global, organized approach applicable to DN as well as to other relevant renal conditions.

Conflict of Interests

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

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Research Article

Association of Haemostatic and Inflammatory Biomarkers with Nephropathy in Type 1 Diabetes Mellitus

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This study aimed at investigating the association between haemostatic biomarkers, proinflammatory, and anti-inflammatory cytokines with chronic kidney disease in type 1 diabetic patients. Patients were divided into two groups: with nephropathy (albuminuria $\geq$ 30 mg/g and/or GFR $<$ 60 mL/min/1.73 m^2), $n = 65$; and without nephropathy (albuminuria $<$ 30 mg/g and GFR $\geq$ 60 mL/min/1.73 m^2), $n = 60$. INF-γ, IL-6, IL-10, and TNF-α plasma levels were determined by flow cytometry. VWF, ADAMTS13 antigen, and D-Dimer plasma levels were determined by enzyme-linked immunosorbent assay and ADAMTS13 activity was assessed by fluorescence resonance energy transfer assay. Elevated levels of INF-γ, VWF, ADAMTS13 antigen, D-Dimer, and reduced ADAMTS13 activity/antigen ratio were observed in patients with nephropathy as compared to those without nephropathy ($P = 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.). Cytokines and haemostatic biomarkers remained associated with nephropathy after adjustments (use of statin, acetylsalicylic acid, angiotensin converting enzyme inhibitor, and angiotensin antagonist). INF-γ, TNF-α, and IL-10 significantly correlated with haemostatic biomarkers. Inflammatory and hypercoagulability status are associated with nephropathy in type 1 diabetes mellitus and an interrelationship between them may play an important role in pathogenesis of diabetic nephropathy.

1. Introduction

Chronic kidney disease (CKD) is a very common complication of diabetes mellitus (DM) [1]. It is defined as the presence of abnormalities in renal structure or function for longer than three months, with implications to healthy. The diagnostic criteria for CKD consist of the presence of one or more biomarkers of kidney parenchyma injury such as albuminuria and/or a glomerular filtration rate (GFR) lower than 60 mL/min/1.73 m^2 over longer than three months [2].

The complications of diabetic nephropathy, such as end stage renal disease and cardiovascular events, are responsible for an increased morbidity and mortality among diabetic patients [3]. There are few available pharmacological therapies for treatment of renal disease [4]. Therefore, it is very important to study molecular and metabolic mechanisms involved with the development and progression of renal

dysfunction in DM, since this could contribute to the development of new therapeutic strategies.

Emerging evidence suggests that inflammatory pathways play an important role in the development and progression of diabetic nephropathy. It has been reported that T cells and inflammatory cytokines exert central roles in the development of renal disease in DM [5]. Moreover, hyperglycemia can induce the expression of several proinflammatory cytokines, such as interferon gamma (INF-γ), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), leading to the development of a chronic subclinical inflammatory status in DM [6]. The deficiency of interleukin-10 (IL-10), an anti-inflammatory cytokine, may also be associated with this increased inflammatory status [7].

The interrelation between inflammation and metabolic abnormalities in DM may lead to endothelial injury and the release of von Willebrand factor (VWF) [6]. Increased levels of VWF, a biomarker of endothelial dysfunction, have been associated with renal disease in type 1 (DM1) and type 2 diabetes mellitus (DM2) [8, 9]. VWF promotes platelet adhesion at vascular damage sites, where it mediates the progression of thrombus formation [10]. Elevated levels of VWF can promote microthrombi formation in vasculature, leading to hypercoagulability status and formation of D-Dimer, which is a fragment of fibrin degradation and has also been associated with diabetic nephropathy [11, 12]. ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13) is an enzyme responsible for cleavage of large multimers of VWF [10]. An imbalance between VWF and ADAMTS13 may also contribute to the development of microvascular complications in diabetic patients [13].

In a previous study, we have described the association between VWF, ADAMTS13, and D-Dimer with different levels of renal dysfunction in DM1 patients and we have raised the hypothesis that inflammation could be associated with hypercoagulability in patients with diabetic nephropathy [14]. Therefore, the aim of the present study was to investigate the association between the haemostatic biomarkers VWF, ADAMTS13, and D-Dimer, the proinflammatory cytokines INF-γ, IL-6, and TNF-α, and the anti-inflammatory cytokine IL-10 with CKD in DM1 patients.

2. Patients and Methods

2.1. Ethics. All procedures performed in this study were in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study was approved by the Research Ethics Committee of Federal University of Minas Gerais (CAAE, 0392.0.203.000-11) and informed consent was obtained from all individual participants included in the study. The research protocol did not interfere with any medical recommendations or prescriptions.

2.2. Patients. The clinical records of all 240 DM1 patients receiving assistance at Endocrinology Outpatient Services of the University Hospital (*Hospital das Clínicas*) and Santa Casa/Belo Horizonte, Brazil, from November 2011 to September 2012, were analyzed. After application of exclusion criteria, 125 patients with clinical and laboratorial diagnosis of DM1, according to American Diabetes Association criteria [15], from 18 to 60 years of age, were selected for this study.

DM1 patients with hepatic disease, alcoholism, haemostatic abnormalities, malignant diseases, acute infectious, pregnancy, undergoing hemodialysis, and history of kidney transplantation and cardiovascular diseases were excluded from the study.

2.3. Study Protocol. A detailed history and clinical variables of each patient were obtained from medical records: age, sex, body mass index (BMI), diabetes duration, presence of diabetes complications such as retinopathy and neuropathy, and use of medicines such as angiotensin converting enzyme inhibitor (ACEi), angiotensin antagonist, thyroxine, statin, and acetylsalicylic acid (AAS).

DM1 patients were divided into two groups: with CKD (albuminuria $\geq$ 30 mg/g and/or GFR < 60 mL/min/1.73 m^2), n = 65; and without CKD (albuminuria < 30 mg/g and GFR $\geq$ 60 mL/min/1.73 m^2), n = 60. The presence of increased albuminuria and reduced GFR was confirmed in two out of three occasions, over a period between three and six months [2].

2.4. Determination of Biochemistry Parameters. HbA1c was determined by immunoturbidimetric method in EDTA whole blood samples and creatinine was determined by enzymatic method in serum samples, using Johnson & Johnson dry chemistry technology kits (Ortho Clinical Diagnostics) and VITROS 4600 analyser. The GFR was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula based on creatinine [16].

Urinary albumin excretion (UAE) was determined in urine samples collected after at least 4 hours of urinary retention in the morning, and urinary albumin was normalized by urinary creatinine. Urinary albumin was evaluated by immunoturbidimetric method and urinary creatinine was assessed by enzymatic method, using Johnson & Johnson dry chemistry technology kits (Ortho Clinical Diagnostics) and VITROS 4600 analyser.

2.5. Inflammatory Cytokines Measurement. INF-γ, IL-6, IL-10, and TNF-α plasma levels were determined by flow cytometry, using *Human Basic Kit FlowCytomix* (eBioscience), following the manufacturer's recommendations. Data acquisition and analysis were performed in dual-laser FACS caliber TM flow cytometer (BD Biosciences Pharmingen, San Jose, CA, USA), using the BD Bioscience CBA software.

2.6. Haemostatic Biomarkers Measurement. VWF, ADAMTS13 antigen, and D-Dimer plasma levels were determined by enzyme-linked immunosorbent assay (ELISA), using IMUBIND VWF kit (American Diagnostica), IMUBIND ADAMTS13 kit (American Diagnostica), and ASSERACHROM D-Di kit (StagoDiagnostica), respectively. ADAMTS13 activity was assessed by fluorescence resonance energy transfer (FRET) assay, using ACTIFLUOR ADAMTS13 activity kit (Sekisui Diagnostics). Intra- and

Table 1: Characteristics of type 1 diabetic patients with and without chronic kidney disease.

	Patients without CKD	Patients with CKD	P
Number of individuals (n)	60	65	
Age (years)	32 (25–37)	34 (27–43)	NS
Sex/male (n, %)	23 (38.3)	22 (33.8)	NS
BMI (kg/m^2)	24 ± 3	23 ± 2	$P = 0.030$
Diabetes duration (years)	18 ± 8	19 ± 6	NS
Retinopathy (n, %)	16 (26.7)	39 (60.0)	$P < 0.001$
Neuropathy (n, %)	11 (18.3)	9 (13.8)	NS
Use of ACEi or angiotensin antagonist (n, %)	29 (48.3)	44 (67.7)	$P < 0.001$
Use of statin (n, %)	10 (16.7)	30 (46.2)	$P < 0.001$
Use of AAS (n, %)	3 (5.0)	18 (27.7)	$P = 0.001$
Use of thyroxine (n, %)	6 (10.0)	12 (18.5)	NS
HbA1c (%)	8.4 ± 1.6	8.7 ± 1.4	NS
Creatinine (mg/dL)	0.79 (0.66–0.88)	1.07 (0.76–1.49)	$P < 0.001$
UAE (mg/g of creatinine)	6 (4–14)	65 (38–141)	$P < 0.001$
GFR (mL/min/1.73 m^2)	114 (104–123)	75 (44–106)	$P < 0.001$
INF-γ (pg/mL)	95 ± 20	119 ± 45	$P = 0.001$
TNF-α (pg/mL)	166 (104–215)	215 (149–314)	$P = 0.004$
IL-6 (pg/mL)	16 (14–18)	17 (15–19)	$P = 0.016$
IL-10 (pg/mL)	1106 (1019–1295)	1236 (1024–1523)	$P = 0.030$
VWF (mU/mL)	1028 ± 287	1350 ± 414	$P < 0.001$
ADAMTS13 antigen (ng/mL)	305 (231–509)	549 (357–638)	$P < 0.001$
ADAMTS13 activity (%)	95 ± 16	105 ± 21	$P = 0.003$
VWF/ADAMTS13 antigen	3.1 (1.9–4.2)	2.6 (1.9–3.3)	NS
VWF/ADAMTS13 activity	11.1 ± 3.8	13.2 ± 4.4	$P = 0.006$
ADAMTS13 activity/antigen	0.31 (0.20–0.40)	0.18 (0.17–0.19)	$P < 0.001$
D-Dimer (ng/mL)	191 (137–258)	309 (202–451)	$P < 0.001$

Normally distributed data were expressed as mean ± SD and compared by t-test. Not normally distributed data were expressed as median (percentiles 25%–75%) and compared by Mann-Whitney U test. Categorical variables were expressed as frequencies n (%) and compared using the chi-square test (χ^2). Body mass index (BMI), time of diagnosis, HbA1c, interferon gamma (INF-γ), von Willebrand factor (VWF), ADAMTS13 activity, and VWF/ADAMTS13 activity ratio were normally distributed. Age, creatinine, urinary albumin excretion (UAE), glomerular filtration rate (GFR), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), ADAMTS13 antigen, VWF/ADAMTS13 antigen ratio, ADAMTS13 activity/antigen ratio, and D-Dimer were not normally distributed. NS = not significant. CKD = chronic kidney disease. AAS = acetylsalicylic acid. ACEi = angiotensin converting enzyme inhibitor.

interassay coefficients of variations were, respectively, 9% and 13% for VWF, 4.0% and 7.3% for ADAMYS13 antigen, <6% and <10% for D-Dimer, and 4.1% and 4.4% for ADAMTS13 activity.

2.7. Statistical Analysis. Statistical comparisons were performed using SPSS software (version 20.0, SPSS). Shapiro-Wilk test was used to test if continuous variables were normally distributed. Data normally distributed were expressed as mean ± SD and were compared by Student's t-test. Data not normally distributed were expressed as median (percentiles 25%–75%) and were compared by Mann-Whitney U test. Categorical variables were expressed as frequencies and compared using chi-square test (χ^2). Association between haemostatic and inflammatory biomarkers with CKD was evaluated by bivariate and multivariate logistic regression analysis and odds ratio was calculated. Variables included in multivariate logistic regression analysis were previously associated with CKD in bivariate logistic regression analysis ($P < 0.2$) and consisted of use of ACEi or angiotensin antagonist, use of statin, and use of AAS. Correlations between proinflammatory cytokines and the anti-inflammatory ones and between inflammatory and haemostatic biomarkers were performed using Spearman correlation test. Differences were considered significant when $P \leq 0.05$.

3. Results

Characteristics and clinical variables of 125 DM1 patients included in this cross-sectional study are presented in Table 1. Patients with CKD had lower BMI ($P = 0.030$) and a higher frequency of use of IECA or angiotensin antagonist ($P < 0.001$), statin ($P < 0.001$), and AAS ($P = 0.001$) than patients without CKD. There were no significant differences between the groups regarding age, sex, diabetes duration, HbA1c, use of thyroxine, and presence of neuropathy. However, a higher frequency of retinopathy in group of patients with nephropathy as compared to patients without CKD ($P < 0.001$) was observed.

Table 2: Association between haemostatic and inflammatory biomarkers with chronic kidney disease in type 1 diabetic patients.

Variable	Odds ratio (95% confidence interval) unadjusted	P	Odds ratio (95% confidence interval) adjusted	P
INF-γ	1.021 (1.008–1.035)	0.002	1.025 (1.010–1.040)	0.001
TNF-α	1.006 (1.002–1.011)	0.002	1.008 (1.003–1.013)	0.001
IL-6	1.224 (1.051–1.426)	0.009	1.304 (1.091–1.559)	0.004
IL-10	1.002 (1.000–1.003)	0.018	1.003 (1.001–1.004)	0.003
VWF	1.003 (1.001–1.004)	<0.001	1.003 (1.001–1.004)	<0.001
ADAMTS13 antigen	1.005 (1.002–1.007)	<0.001	1.005 (1.002–1.007)	<0.001
ADAMTS13 activity	1.030 (1.009–1.051)	0.004	1.034 (1.011–1.057)	0.004
VWF/ADAMTS13 antigen	0.667 (0.490–0.907)	0.010	0.702 (0.501–0.984)	0.040
VWF/ADAMTS13 activity	1.129 (1.031–1.236)	0.009	1.125 (1.017–1.244)	0.022
ADAMTS13 activity/antigen	7×10^{-11} (4×10^{-16}–1×10^{-5})	<0.001	7×10^{-11} (4×10^{-16}–1×10^{-5})	<0.001
D-Dimer	1.008 (1.004–1.012)	<0.001	1.008 (1.004–1.012)	<0.001

Data was evaluated by bivariate and multivariate logistic regression analysis and are presented as odds ratio (95% confidence interval) per unit increase of exposure variable. Variables included in multivariate logistic regression analysis were previously associated with chronic kidney disease in bivariate logistic regression analysis ($P < 0.2$) and consisted of use of angiotensin converting enzyme inhibitor (ACEi) or angiotensin antagonist, use of statin, and use of acetylsalicylic acid (AAS).

INF-γ, TNF-α, IL6, and IL-10 plasma levels were higher in patients with CKD as compared to those without nephropathy ($P = 0.001$, $P = 0.004$, $P = 0.016$, and $P = 0.030$, resp.) (Table 1). Patients with CKD also presented elevated plasma levels of VWF, ADAMTS13 antigen, ADAMTS13 activity, D-Dimer and VWF/ADAMTS13 activity ratio, and a reduced ADAMTS13 activity/antigen ratio than those without nephropathy ($P < 0.001$, $P < 0.001$, $P = 0.003$, $P < 0.001$, $P = 0.006$, and $P < 0.001$, resp.). VWF/ADAMTS13 antigen ratio was not significantly different between the groups.

The results of the bivariate logistic regression analysis are presented in Table 2. In general, inflammatory and haemostatic biomarkers were significant associated with CKD, corresponding to OR of 1.021 (1.008–1.035) for INF-γ, 1.006 (1.002–1.011) for TNF-α, 1.224 (1.051–1.426) for IL-6, 1.002 (1.000–1.003) for IL-10, 1.003 (1.001–1.004) for VWF, 1.005 (1.002–1.007) for ADAMTS13 antigen, 1.030 (1.009–1.051) for ADAMTS13 activity, 0.667 (0.490–0.907) for VWF/ADAMTS13 antigen, 1.129 (1.031–1.236) for VWF/ADAMTS13 activity, 7×10^{-11} (4×10^{-15}–1×10^{-5}) for ADAMTS13 activity/antigen, and 1.008 (1.004–1.012) for D-Dimer. These associations remained significant even after adjustment for use of ACEi or angiotensin antagonist, statin, and AAS, as shown by multivariate regression logistic analysis (Table 2).

All proinflammatory cytokines IL-6, INF-γ, and TNF-α presented a significant correlation ($P < 0.001$) with the anti-inflammatory cytokine IL-10 ($R^2 = 0.689$, 0.805, and 0.813, resp.), as presented in Figure 1. Correlations between inflammatory and haemostatic biomarkers are presented in Table 3. INF-γ presented a significant correlation with VWF, ADAMTS13 antigen, VWF/ADAMTS13 activity ratio, and ADAMTS13 activity/antigen ratio ($R = 0.264$, 0.192, 0.220, and −0.385, resp.). TNF-α was significantly correlated with ADAMTS13 antigen, ADAMTS13 activity/antigen ratio, and D-Dimer ($R = 0.291$, −0.337, and 0.217, resp.). IL-10 showed a significant correlation with VWF, VWF/ADAMTS13 activity ratio, ADAMTS13 activity/antigen ratio, and D-Dimer ($R = 0.212$, 0.192, −0.270, and 0.244, resp.). IL-6 was not significantly correlated with none of the haemostatic biomarkers.

4. Discussion

In our study, higher levels of INF-γ, IL-6, and TNF-α were observed in DM1 patients with CKD in comparison to those without nephropathy, which is in agreement with other studies [17–22].

Hyperglycemia is associated with increased production of advanced glycation end-products (AGEs), which can bind to their receptors present on the surface of endothelial cells, smooth muscle cells, fibroblasts, lymphocytes, monocytes, and macrophages, resulting in activation of NF-κB which can enhance INF-γ transcription in T cells and IL-6 and TNF-α transcription in diabetic glomerulus [23–25]. Large amounts of INF-γ are produced by T helper 1 (Th1) cells, promoting activation of macrophages and cell-mediated immunity, which may mediate tissue injury in diabetic patients [26]. TNF-α can induce monocyte chemoattractant protein-1 (MCP-1) expression in mesangial cells, resulting in macrophage recruitment and accumulation in glomerulus, exacerbating kidney inflammation [27]. Hyperglycemia also causes enhanced cycle oxygenize-2 (COX-2) expression and prostaglandin E2 (PGE2) production, which induces IL-6 expression in tubular epithelial cells, contributing to the development of glomerulosclerosis, interstitial fibrosis, and albuminuria [28]. Furthermore, IL-6 and TNF-α are cytotoxic cytokines and may contribute to glomerulus endothelial cells damage, which can stimulate the release and expression of procoagulant molecules, such as VWF, plasminogen activator inhibitor-1 (PAI-1), and tissue factor. They can also inhibit the expression of anticoagulant molecules, such

Table 3: Correlations between haemostatic and inflammatory biomarkers in type 1 diabetic patients.

	VWF	ADAMTS13 antigen	ADAMTS13 activity	VWF/ADAMTS13 antigen	VWF/ADAMTS13 activity	ADAMTS13 activity/antigen	D-Dimer
INF-γ	0.264**	0.192*	0.046	−0.125	0.220**	−0.385**	0.105
TNF-α	0.183	0.291**	0.127	−0.158	0.073	−0.337**	0.217*
IL-6	0.082	0.083	−0.007	−0.044	0.069	−0.130	0.162
IL-10	0.212*	0.103	0.013	−0.003	0.192*	−0.270*	0.244*

Correlations were performed using Spearman correlation test. Data was expressed as correlation coefficient (R).
*Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level.

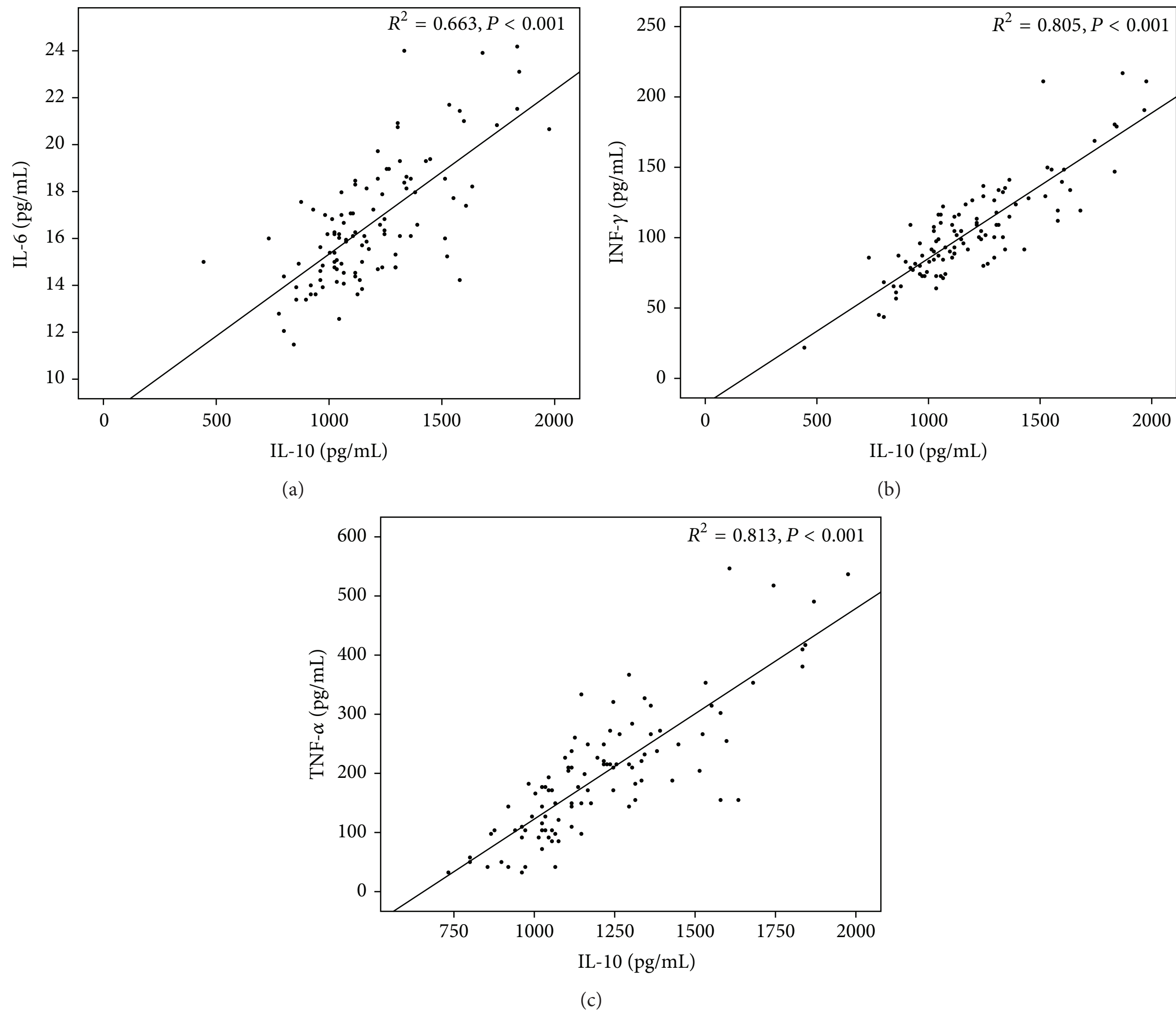

Figure 1: Correlation between IL-6 (a), INF-γ (b), and TNF-α (c) with IL-10 in type 1 diabetic patients.

as thrombomodulin, by endothelial cells [29], resulting in hypercoagulability state, which may also contribute to progression of diabetic nephropathy [8].

Wolkow et al. [30] reported that IL-6 may be associated with the progression of nephropathy in DM1 patients with microalbuminuria and a meta-analysis has demonstrated that IL-6 is a valid biomarker predicting the progression of nephropathy in DM2 [31]. Moreover, urinary TNF-α and soluble TNF receptors 1 and 2 (TNFR1 and TNFR2) have been shown to be significant predictors of GFR decline in DM2 patients [32, 33]. Therefore, it is possible to suggest that hyperglycemia promotes the development of a chronic low grade inflammation in renal parenchyma, characterized by increased levels of proinflammatory cytokines INF-γ, TNF-α, and IL-6 levels, which may be involved in the pathogenesis of diabetic nephropathy.

IL-10 is produced by T helper 2 (Th2) cells and plays an important role in limiting the cascade of proinflammatory cytokines activation and downregulating T cell-mediated immune responses [34]. Treatment of rats with IL-10 reduced proteinuria and inflammatory status and attenuated renal injury [35]. A lower frequency of CG genotype polymorphism IL-10 -1082G/A, which is related to higher expression of this cytokine, was associated with nephropathy in DM2 [36]. In our study, patients with CKD presented higher levels of IL-10 than patients without nephropathy, which is in agreement with other study [34]. This result could be partially explained by a compensatory mechanism, in which IL-10 plasma levels increase as a consequence of higher plasma levels of proinflammatory cytokines INF-γ, TNF-α, and IL-6 in patients with nephropathy to regulate the inflammatory status, since IL-10 is an anti-inflammatory cytokine. The significant correlation between proinflammatory cytokines and IL-10 observed in this study may corroborate this hypothesis. This compensatory increase in IL-10 levels could also prolong the course of nephropathy in DM1 patients.

Elevated plasma levels of VWF were associated with diabetic nephropathy in the present study, which was also verified by other authors [9, 37]. It was demonstrated that some inflammatory cytokines, such as INF-γ, IL-6, and TNF-α, can promote the release of VWF [29, 38]. Indeed, a significant correlation between VWF and INF-γ was observed in this study, which indicates that the interrelation between inflammation and endothelial dysfunction may be involved in the pathogenesis of diabetic nephropathy.

ADAMTS13 antigen and activity plasma levels were also elevated in DM1 patients with CKD as compared to those without renal dysfunction, which was also found in other studies [39]. This result may be explained by a compensatory mechanism, by which ADAMTS13 synthesis is increased due to the marked elevation in VWF plasma levels, keeping VWF/ADAMTS13 antigen ratio unchanged. However, the rise in ADAMTS13 antigen levels was not accompanied by a proportional increase in ADAMTS13 activity, since ADAMTS13 activity/ADAMTS13 Ag ratio was lower and VWF/ADAMTS13 activity ratio was higher in DM1 patients with nephropathy.

An *in vitro* study has demonstrated that IL-6 can inhibit ADAMTS13 activity [40], compromising the proteolysis of VWF. TNF-α and INF-γ presented a significant negative correlation with ADAMTS13 activity/antigen ratio and a significant positive correlation with ADAMTS13 antigen, but not with ADAMTS13 activity, which is in agreement with the hypothesis that the inflammatory status induced by hyperglycemia may also result in reduced activity of ADAMTS13 and, consequently, in reduced proteolysis of VWF, leading to an imbalance between VWF and ADAMTS13 activity in DM1 patients with CKD. This imbalance may promote microthrombi formation and hypercoagulability status, resulting in elevated D-Dimer plasma levels, which was also observed in DM1 with CKD in this study and other studies [11, 12]. A significant positive correlation between D-Dimer and TNF-α observed in the present study reinforces the link between inflammation and hypercoagulability.

Increased proinflammatory cytokines plasma levels have been associated with the development of cardiovascular disease in DM1 patients [21]. As these cytokines can promote the release of procoagulant molecules, such as VWF [29], and possibly can reduce ADAMTS13 activity, promoting an imbalance between VWF and ADAMTS13 activity and, consequently, microthrombi formation, it is possible to suggest that the chronic low grade inflammation present in patients with CKD may be, at least partially, responsible for the link between diabetic nephropathy and high risk of cardiovascular outcomes. However, longitudinal studies are still necessary to elucidate this issue.

In conclusion, increased plasma levels of proinflammatory cytokines INF-γ, TNF-α, and IL-6, anti-inflammatory cytokine IL-10, and haemostatic biomarkers VWF, ADAMTS13 antigen, ADAMTS13 activity, and D-Dimer are associated with CKD in DM1 patients, and the imbalance between VWF/ADAMTS13 activity and ADAMTS13 activity/antigen is correlated with inflammatory cytokines, suggesting that an interrelation between inflammation and hypercoagulability may contribute to the development and progression of renal disease in DM1. These findings may be useful for guiding future longitudinal studies aiming at a better comprehension of the interrelation between inflammation and hypercoagulability with diabetic nephropathy progression, enabling the identification of new biomarkers of renal disease progression and the introduction of new therapeutic strategies.

Conflict of Interests

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

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Review Article

Review of Herbal Traditional Chinese Medicine for the Treatment of Diabetic Nephropathy

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Diabetic nephropathy (DN) is the most serious chronic complications of diabetes; 20–40% of diabetic patients develop into end stage renal disease (ESRD). However, exact pathogenesis of DN is not fully clear and we have great difficulties in curing DN; poor treatment of DN led to high chances of mortality worldwide. A lot of western medicines such as ACEI and ARB have been demonstrated to protect renal function of DN but are not enough to delay or retard the progression of DN; therefore, exploring exact and feasible drug is current research hotspot in medicine. Traditional Chinese medicine (TCM) has been widely used to treat and control diabetes and its complications such as DN in a lot of scientific researches, which will give insights into the mechanism of DN, but they are not enough to reveal all the details. In this paper, we summarize the applications of herbal TCM preparations, single herbal TCM, and/or monomers from herbal TCM in the treatment of DN in the recent 10 years, depicting the renal protective effects and the corresponding mechanism, through which we shed light on the renal protective roles of TCM in DN with a particular focus on the molecular basis of the effect and provide a beneficial supplement to the drug therapy for DN.

1. Introduction

Diabetic nephropathy (DN) is a widely recognized microvascular complication of diabetes and almost the leading cause of end-stage kidney failure worldwide responsible for morbidity and mortality [1]. Clinical manifestations of DN include initial increase in glomerular filtration (GFR), proteinuria, increased creatinine levels, and eventually decreased GFR [2–4]. Major pathological changes of DN are virtually indistinguishable in both type 1 and type 2 diabetes, including mesangial expansion, extracellular matrix (ECM) accumulations, tubulointerstitial fibrosis, and glomerular sclerosis. Hyaline arteriolosclerosis is often prominent in the established DN pathological features caused by endothelial dysfunction and inflammation [5–7].

Multiple factors have been implicated in the pathogenesis of DN including hyperglycemia induced activation of advanced glycation end products (AGEs) and reactive oxygen species (ROS); JAK-STAT pathways and G protein signaling; activation of the PKC, renin-angiotensin aldosterone system (RAAS), transforming growth factor β-Smad-mitogen-activated protein kinase (TGF-β-Smad-MAPK), deregulated expression of cyclin dependent kinases (CDK), and their inhibitors; and aberrant expression of ECM proteins, ECM-degrading enzymes, metalloproteinases, and their inhibitors [8]. The abovementioned factors can induce aberrant expression of profibrotic and proinflammatory cytokines, cell-cycle genes, and ECM genes involved in DN [9]. A large number of novel treatment options has arisen from experimental studies based on the pathogenic factors of DN, including intensive glycemic control, precise blood pressure control, optimal RAAS blockade with ACEI/ARB, life style modifications such as exercise and dietary restrictions, and a lot of novel agents [10], but the portion of ESRD due to DN still remains high in spite of the widespread application of numerous therapeutic approaches focusing on the management of factors mentioned above [11–13]. Therefore, interventions that could effectively delay the progression of DN are greatly required.

In China, traditional Chinese medicine (TCM) has been widely used in the treatment of diabetes and its complications

for a long time [14]; TCM has lots of advantages over the conventional medical approaches in the prevention of diabetic complications because of less toxicity and/or side effects [15–17]. In this review, we will explore the advance of herbal TCM treatment on DN in recent 10 years, based on the experimental and clinical studies to note the scientific basis for the therapeutic effects of TCM on DN.

2. Applications of TCM in DN

Plants have been widely used for medical purposes long before recorded history [18]. In China, TCM emerged and influenced the surrounding countries such as Japan and South Korea; increasing popularity of TCM caused great interests in laboratory and clinical investigations in lots of diseases on its efficiency and action mechanism. TCM manifests as herbal medicine, acupuncture, moxibustion, massage, dietary therapy, and physical exercise including shadow boxing and Qigong, and herbal remedies are the focus of TCM in mainland China [19] and acupuncture is prevalent in the United States [18]. Under the urgent need for the treatment of DN, we focus on the update of the efficient herbal TCM preparations, single herbal TCM, and/or monomers from herbal TCM in DN related clinical and experimental trials, through which we explore the effective herbal TCM for DN and clearly put forward underlying mechanism in the treatment of DN.

2.1. TCM Preparations in DN. TCM preparations are applied as decoction, pill, and capsule in the treatment of DN. We will introduce the TCM preparations in alphabetical order about components of TCM preparations, therapeutic effects in clinical or experimental studies, and relevant mechanism. All the mentioned TCM preparations in this review are listed in Table 1.

2.1.1. Chaihuang Yishen Granule (CHYS). Chaihuang Yishen granule (CHYS, also called Qilong-Lishui granule) is composed of radix astragali, *Dioscorea nipponica*, radix bupleuri, *Angelica sinensis*, *Pyrrosia petiolosa*, *Polyporus umbellatus*, and *Hirudo nipponica*. A recent study in STZ plus uninephrectomized induced rats showed that CHYS could be a therapeutic agent for DN by blocking TGF-β/Smad3-mediated renal fibrosis [20].

2.1.2. Compound Rhizoma Coptidis Capsule (CRCC). Compound rhizoma coptidis capsule (CRCC) is composed of rhizoma coptidis, Kudzu root, dwarf lilyturf,and Loquat leaf. CRCC has been shown to protect renal function and slow down the progression of DN by the suppression of TGF-β1 and type IV collagen expression in STZ induced diabetic rats [21].

2.1.3. Compound Shenhua Tablet (CST). Compound Shenhua Tablet (CST), is composed of radix astragali, fructus ligustri lucidi, rhizoma *zedoaria*, and honeysuckle. CST treatment in STZ induced diabetic rats showed that urine mAlb, Scr, BUN, Glu, TG, and TC were significantly lower than the diabetic model group [22].

2.1.4. Danggui Buxue Tang (DBT). Danggui buxue tang (DBT), a preparation including radix astragaliandradix *Angelica sinensis*, has been shown to partially attenuate the increases in blood glucose, TG, and CHO, and DBT was supposed to retard DN progression by suppressing TGF-β1 expression in STZ induced diabetic rats [23]. In the HG stimulated glomerular mesangial cells, DBT could inhibit cell proliferation and expression of LN, FN, and collagen IV indicating the renoprotective effect of DBT on DN at the early stages [24].

2.1.5. Danggui Shaoyao San (DSS). Danggui Shaoyao San (DSS) is a famous TCM formula comprising six herbal medicines: radix Paeoniae Alba, radix *Angelica sinensis*, rhizoma Chuanxiong, *Poria cocos*, rhizoma *Atractylodis macrocephala*, and rhizoma Alismatis. DSS has been shown to protect renal function in STZ induced diabetic rats through regulating plasma glucose and attenuating AGEs expression in diabetic glomeruli [25].

2.1.6. Fufang Xue Shuan Tong (FXST). Fufang Xue Shuan Tong (FXST) capsule is composed of radix notoginseng, *Salvia miltiorrhiza*, XuanShen, and radix astragali and has been used to treat DN for many years. High dose of FXST treatment could prevent glomerular hypertrophy and mesangial matrix expansion through regulation of oxidative stress including increasing SOD activities and decreasing MDA levels in the kidney of HFD-fed plus STZ induced rats [26].

2.1.7. Hachimijiogan (HJG). A most popular herbal medicine in Japanese Kampo, Hachimijiogan (HJG, Ba Wei Di Huang Wan in Chinese), is extracted from a mixture of *Rehmannia* radix, corni fructus, *Dioscorea* rhizome, Hoelen, Alismatis rhizome, Moutan cortex, Cinnamomi cortex, and Aconiti tuber. In subtotal nephrectomy plus STZ induced rats, HJG could reduce blood glucose and urinary protein excretion levels and increase Ccr; furthermore, HJG could ameliorate oxidative stress and AGEs formation associated with DN and subsequently prevent the development of renal lesions including glomerular sclerosis, tubulointerstitial lesions, mesangial expansions, and atherosclerosis [27]. In spontaneous diabetic WBN/Kob rats with DN, HJG could prevent DN progression through several established biomarkers in plasma [28] and by reducing renal oxidative injury and expression of FN and TGF-β1 proteins [29]. In OLETF rats, HJG could reduce TGF-β1, FN, iNOS, and COX-2 expressions in kidney cortex, urinary protein excretion was decreased, Ccr levels were improved, and serum glycosylated protein and AGEs were reduced effectively; data mentioned above suggested that HJG has beneficial effect on the DN progression [30].

2.1.8. Hu-Lu-Ba-Wan (HLBW). Hu-Lu-Ba-Wan (HLBW), composed of *Trigonella foenum-graecum* L. (*TFG*) and *Psoralea corylifolia* L. (*PC*), has been shown to improve hyperglycemia, hyperlipidemia, and proteinuria in the HFD-fed plus STZ induced rats and could play renoprotective effect in attenuating renal oxidative stress via PKC-α/NADPH oxidative pathway [31].

Table 1: Applications of herbal TCM Preparations in DN.

Name	Origins	Methods	Results	Pathways
CHYS	Radix astragali, *Dioscorea nipponica*, radix bupleuri, *Angelica sinensis*, *Pyrrosia petiolosa*, *Polyporus umbellatus*, and *Hirudo nipponica*	*Type 1 diabetic animal study* (STZ + nephrectomized rat)	Inhibiting 24 h proteinuria and progressive renal fibrosis (glomerulosclerosis index, tubulointerstitial fibrosis index, and upregulation of ECM), upregulating Smad7, and downregulating TGF-β1, TGF-βR, Smad3 activation, and miRNA-21	[20]
CRCC	Rhizoma coptidis, Kudzu root, dwarf lilyturf, and loquat leaf	*Type 1 diabetic animal study* (STZ induced rats)	Reducing FBG, BUN, Cr, Upro levels and TGF-β1, and collagen IV expressions and alleviating pathological lesions of kidney	Through TGF-β1 pathway [21]
CST	Radix astragali, fructus ligustri lucidi,Rhizoma *zedoaria*, and honeysuckle	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing urine mAlb, Scr, BUN, Glu, TG, and TC	[22]
DBT	*Angelica sinensis and Astragalus membranaceus*	*Type 1 diabetic animal study* (STZ induced rats)	Attenuating the increases in blood glucose, TG and CHO, and TGF-β1 expression in kidney	Through TGF-β1 way [23, 24]
		Cellular study (mesangial cells)	Inhibit cell proliferation and expression of LN, FN, and collagen IV	
DSS	Radix Paeoniae Alba, radix *Angelica sinensis*, rhizoma *Chuanxiong*, *Poria cocos*, rhizoma *Atractylodis macrocephala*, and Alismatis rhizome	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing FBG and attenuating AGEs expression in diabetic glomeruli	Through modulating oxidative stress via AGEs expression [25]
FXST	SanQi, DanShen, XuanShen, and HuangQi	*Type 2 diabetic animal study* (HFD + STZ induced rats)	Preventing glomerular hypertrophy and mesangial matrix expansion	Through regulating oxidative stress [26]
HJG	*Rehmanniae* radix, *Corni fructus*, *Dioscorea* rhizome, Hoelen, Alismatis rhizome, Moutan cortex, Cinnamomi cortex, and Aconiti tuber	*Type 1 diabetic animal study* (STZ + nephrectomized rat)	Reducing blood glucose and urinary protein excretion and increasing creatinine clearance, ameliorating oxidative stress and AGEs formation associated with DN, and preventing the development of renal lesions including glomerular sclerosis, tubulointerstitial lesions, mesangial expansions, and atherosclerosis	Inhibiting AGEs formation and sorbitol levels in kidney [27]
		Type 1 diabetic animal study (WBN/Kob rats)	Preventing diabetic kidney damage	Reducing renal oxidative injury and expression of FN/TGF-β1 proteins [28, 29]
HLBW	*Trigonella foenum-graecum* L. (*TFG*) and *Psoralea corylifolia* L. (*PC*)	*Type 2 diabetic animal study* (HFD + STZ induced rats)	Improving hyperglycemia, hyperlipidemia, and proteinuria	Through attenuating renal oxidative stress via PKC-α/NADPH oxidative pathway [31]
LDP	*Rehmannia glutinosa*, Cornel (manufactured), Moutancortex, *Yam*, *Poria cocos*, and *Alisma*	*Human study* (DN patients)	Improving symptoms and signs of DN, inhibiting EAR activity, lowering UAER levels, β_2-microglobulin in blood, and urine, and relieving DN	[32, 33]

Table 1: Continued.

Name	Origins	Methods	Results	Pathways
Oryeongsan	*Poria, Alismatis rhizoma, Polyporus umbellatus (Pers.) Fries,* rhizoma *Atractylodis macrocephala*, and Ramulus Cinnamomi Cassiae	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing plasma glucose, UAER, and Ccr, attenuating mesangial matrix expansion, and downregulating increased NF-κB, TGF-β1 expression, elevated AGEs, and FN accumulation	Through attenuating increased NF-κB and TGF-β1 expression [34]
		Type 2 diabetic animal study (db/db mice)	Decreasing TC and TG, improving blood glucose, insulin, glucose tolerance, and HOMA-IR, Ccr, urine albumin, and BUN, and reducing TGF-β1, Smad2/4, collagen IV, CTGF, and TIMP	Through disturbing the TGF-β1/Smads pathway [35]
QJC	Radix astragali, *Hirudo, Rehmannia* root, and rhizoma Polygonati	*Human study* (DN patients)	Decreasing SBP and DBS, increasing ALB, and slowing down the increase of Scr and decrease of eGFR	[36]
QWG	Radix astragali, radix *Rehmanniae, Euonymus alatus*, and Rhubarb	*Type 2 diabetic animal study* (KK-Ay mice)	Alleviate renal pathological changes and decreasing TGF-β1 expression	Through inhibiting TGF-β1 expression [37]
SKW	Radix astragali, Herba Leonuri	*Type 1 diabetic animal study* (STZ induced rats)	Protecting renal function	Through increasing NO and decreasing TGF-β1 excretion; affecting podocytes special proteins expression [38]
		Type 1 diabetic animal study (STZ induced rats)	Alleviating morphological damage of kidney	Through reducing Ang II in plasma and kidney and inhibiting renal AT(1)R [39]
		Cellular study (mesangial cells)	Suppressing FN secretion	Through TGF-β1 way [38]
SQABC	Radix astragali and *Salvia miltiorrhiza*	*Type 1 diabetic animal study* (STZ induced rats)	Reducing 24 h UP excretion and improving reabsorption function	Through enhancing antioxidative activity and upregulating megalin [40, 41]
		Cellular study (NRK-52E cells)	Protecting HG injured NRK-52E cells and improving protein uptake	
TSF	*Astragalus,* raw *Rehmannia* root, sanchi root, euonymus branchlet, rhubarb, bitter orange, and dogwood fruit	*Human study* (DN patients)	Regulating and improving phospholipids metabolism Decreasing in vivo Cys, Hcy, SAM, and SAH	Through inhibiting PKC pathway and reducing phospholipids metabolism; improving in vivo hypomethylation and oxidative stress [42, 43]
		Type 2 diabetic animal study (db/db mice)	Upregulating JAK1, JAK2, and STAT3 and downregulating STAT4	Regulating the JAK/STAT/SOCS pathway [44]
TSL	Radix astragali, radix *Rehmannia,* leech, bile south star, Artemisia anomala, and Ze lan	*Type 1 diabetic animal study* (STZ + nephrectomized rat)	Decreasing ECM components	Through downregulating TGF-β1 and TIMP-2 and upregulating MMP-2 expression [45]

Table 1: Continued.

Name	Origins	Methods	Results	Pathways
TXL	Scorpion, leech, Centipede, groundbeetle, Cicada, Borneol, radix paeoniae rubra, and ginseng	*Human study* (DN patients)	Improving renal function, repairing the renal tubular interstitial damage, and delaying the progression of DN	Through reducing plasma ET-1 and UAER [46]
		Cellular study (HKCs)	Lowering miRNA-21 expression in tissue, serum, and cells, increasing E-cadherin and decreasing α-SMA expression, and decreasing collagen IV and FN and increasing Ccr	Through regulating miRNA-21-induced EMT [47]
		Type 2 diabetic animal study (KK-Ay mice)	Reducing TGF-β1 and Smad3 expressions, restoring Smad7, decreasing collagen IV, FN,and 24 h UAER, BUN, and increasing Ccr	
XCHT	Radixbupleuri, *Scutellaria baicalensis* Georgi radix, *Panax ginseng*, *Pinellia ternata* tuber, *Glycyrrhiza glabra*, Ginger slice, and *Zizyphus vulgaris* Lam. *fructus*	*Type 1 diabetic animal study* (STZ induced rats) *Cellular study* (mesangial cells)	Decreasing the expression of TGF-β1, FN, and collagen IV and increasing BMP-7 expression	Through decreasing oxidative stress and productions of TGF-β1, FN, and collagen IV [48]
XKG	Radix astragali, Mountain *Cornus*, leech, and winged euonymus twig	*Type 1 diabetic animal study* (STZ + nephrectomized rat) *Cellular study* (mesangial cells)	Decreasing fasting blood pressure and urinary protein in 24 hrs Inhibiting high glucose induced RMC proliferation	Through downregulating TGF-β1 expression [49, 50]
XXD	Radix et rhizoma rhei, rhizoma coptidis, and radix *Scutellaria*	*Type 2 animal studies* (HFD + STZ induced rats, db/db mice)	Attenuating albuminuria and renal pathological changes, reducing AGEs, and inhibiting RAGE and inflammation factors expression	Through downregulating NF-κB pathway and reducing renal AGEs and RAGE [51, 52]
XZT	Radix astragali, radix *Rehmannia*, fructus ligustri lucidi, *Scutellaria baicalensis* Georgi, rhizoma coptidis, dodder weed, fairy spleen, and *Salvia miltiorrhiza*	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing blood glucose and HbA1C, improving renal function, ameliorating proteinuria, and reducing glomerular extracellular matrix expansion	Through inhibiting AGEs accumulation and RAGE mRNA levels renal cortex [53]
ZDP	Rhizoma anemarrhenae, cortex phellodendri, radix *Rehmannia* preparata, rhizoma *dioscorea*, fructus corni, Moutan cortex, *Alismatis* rhizoma, and *Poria*	*Type 1 diabetic animal study* (STZ induced rats)	Ameliorating DN	Through inhibiting glucose and lipid metabolism and enhancing methylamine metabolism [54]
ZHM	*Sargassum* and rhizoma rhei	*Cellular study* (human mesangial cells)	Preventing the process of DN	Through decreasing TGF-β1 and collagen IV expression [55]
ZQR	fructus ligustri lucidi, *Eclipta prostrata*, and *Dioscorea opposita*	*Type 2 diabetic animal study* (HFD + STZ induced rats)	Inhibiting TGF-β1 and FN overexpression in the renal cortex	Through inhibiting SREBP-1c overexpression and its target [17]
ZSTL	Raw *Astragalus*, *Angelica*, safflower, zedoary turmeric, dodder, radix *Rehmannia*, dogwood, *Poria*, *Epimedium*, earthworm, and *Schisandra*	*Human study* (DN patients)	Improving HbA1c and FBG, TC, TG, UAER, Scr, ANP, ET-1, and VEGF	Through modifying ANP, ET-1, and VEGF [56]

2.1.9. Liuwei Dihuang Pill (LDP). Liuwei Dihuang Pill (LDP), one formulation in the ancient Chinese medicine, includes six crude drugs: *Rehmannia glutinosa*, fructus corni, cortex Mountain, *Dioscorea opposita*, *Poria cocos*, and *Alisma*. A previous study in DN patients showed that LDP could improve symptoms and signs of DN and inhibit erythrocyte aldose reductase (EAR) activity and lower UAER levels, β_2-microglobulin in blood and urine without affecting blood glucose, lipids, and mean arterial pressure [32]. LDP treatment in type 2 diabetic patients was found to be associated with the relief of DN [33]. Liuwei Dihuang (LW) decoction has also been proven to relieve early DN abnormalities mediated by suppression of renal entothelin-1-reactive oxidative species (ET-ROS) system and escalating MMPs activity [57], and LW without fructus corni could alleviate DN by combined suppression of ET-ROS axis with modulating hypoglycemic effects in STZ induced diabetic rats [58].

2.1.10. Oryeongsan (Wulingsan). Oryeongsan (Wulingsan), also named as Hoelen Five Herb Formula, is composed of five crude drugs: *Poria*, Alismatis rhizoma, *Polyporus umbellatus (Pers.) Fries*, rhizoma *Atractylodis macrocephala*, and Ramulus Cinnamomi Cassiae. A previous study showed that Oryeongsan could play renal protective roles in lowering plasma glucose and ameliorating glycation-mediated renal damage through attenuating increased NF-κB and TGF-β1 expression in STZ induced diabetic rats [34]. Further study showed that Oryeongsan could ameliorate insulin resistance and DN in db/db mice by disturbing the TGF-β1/Smads pathway [35].

2.1.11. Qizhi Jiangtang Capsule (QJC). Qizhi Jiangtang Capsule (QJC) is composed of four crude drugs: radix astragali, *Hirudo*, *Rehmannia* root, and rhizoma Polygonati. In a multicenter randomized clinical study, QJC has been shown to reduce urinary protein effectively and delay the progression of renal function in treating 3b DN patients [36].

2.1.12. Qiwei Granule (QWG). Qiwei Granule (QWG) is composed of radix astragali, radix *Rehmannia*, *Euonymus alatus*, and Rhubarb. QWG could alleviate renal pathological changes and decrease TGF-β1 expression in the type 2 diabetic KK-Ay mice, which suggested that QWG could play roles in preventing and curing DN [37].

2.1.13. Shenkangwan (SKW). Shenkangwan (SKW) is composed of two crude drugs: radix astragaliand Herba Leonuri. SKW was reported to protect renal function by increasing NO production and decreasing TGF-β1 excretion in the mesangial cells from diabetic rats [38]; in diabetic rats SKW could reduce FN expression in kidney [59] while in rat mesangial cells SKW has been shown to suppress FN secretion via TGF-β1 signal way [60]. Another study showed that in STZ induced diabetic rats SKW could protect renal function and alleviate the functional and structural damage of podocytes possibly by reducing desmin and increasing podocin expression [61], and SKW could offer renal protection against DN by reducing Ang II levels in the plasma and kidney tissues and inhibiting renal AT(1)R expressions [39]. All the data supply precise mechanism of SKW treating DN.

2.1.14. Supplementing Qi and Activating Blood Circulation (SQABC). Supplementing Qi and activating blood circulation (SQABC) is composed of radix astragali and *Salvia miltiorrhiza* and has been shown to reduce 24 h urinary protein excretion and improve tubular reabsorption function by enhancing renal tissue activity of antioxidant and upregulating megalin expression in tubular epithelial cells in STZ induced diabetic rats [40]. Another *in vitro* study showed that supplementing Qi and activating blood circulation could protect HG injured NRK-52E cells and improve protein uptake by increasing megalin expression [41].

2.1.15. Tangshen Formula (TSF). Tangshen Formula (TSF) is composed of *Astragalus*, raw *Rehmannia* root, sanchi root, *Euonymus* branchlet, rhubarb, bitter orange, and dogwood fruit. TSF has been shown to regulate and improve phospholipids metabolism in DN patients related with inhibition of PKC pathway and the corresponding reduction of phospholipase A2 activity [42]. In a study on the Hcy metabolism of DN patients, TSF could improve *in vivo* hypomethylation and oxidative stress showing similar favorable effect to western medicine in the treatment of DN [43]. In the molecular mechanism study using a db/db mice model, TSF showed beneficial effects on DN treatment via regulating the JAK/STAT/SOCS signaling pathway [44].

2.1.16. Tongshenluo (TSL) Capsule. Tongshenluo (TSL) capsule is composed of six crude drugs: radix astragali, radix *Rehmannia*, leech, bile south star, *Artemisia anomala*, and Ze lan. TSL has been shown to decrease the levels of FBG, HbA1c, and urinary mAlb in the subtotal nephrectomy plus STZ induced diabetic rats and decrease the components of ECM through downregulating TGF-β1 and TIMP-2 and upregulating MMP-2 expression [45].

2.1.17. Tongxinluo (TXL). Tongxinluo (TXL) capsule include 8 crude drugs: scorpion, leech, *centipede*, *ground beetle*, *cicada*, *borneol*, radix paeoniae rubra, and ginseng. TXL capsule has been shown to improve renal function, repair the renal tubular interstitial damage, and delay the progression of DN patients by reducing plasma ET-1 and UAER [46]. TXL was also demonstrated to ameliorate renal function and structure by regulating miRNA-21-induced EMT, suggesting miRNA-21 may be one of the therapeutic targets for TXLC in DN [47]. Another study showed that TXL could also successfully inhibit TGF-β1 induced EMT in DN [62].

2.1.18. Xiao Chai Hu Tang (XCHT). Xiao Chai Hu Tang (XCHT, Shosaiko-to in Japanese) is a herbal drug formula extensively applied in TCM and Japanese Kampo medicine, comprising seven medicinal plants: radix bupleuri, *Scutellaria baicalensis* Georgi radix, *Panax ginseng*, *Pinellia ternata* tuber, *Glycyrrhiza glabra*, ginger slice, and *Zizyphus vulgaris* Lam. *fructus*. XCHT has been shown to decrease the expression of TGF-β1, FN, and collagen IV accompanied with increased BMP-7 expression in STZ induced diabetic

mice and HG stimulated RMC, which was mediated through decreasing oxidative stress and productions of TGF-β1, FN, and collagen IV in renal cortex during the development of DN [48].

2.1.19. Xiaoke Granule (XKG). Xiaoke granule (XKG, Xiaoke Keli in Chinese) includes four crude drugs: radix astragali, Mountain *Cornus*, leech, and winged euonymus twig. It was reported that XKG could decrease fasting blood pressure and 24 h urinary protein excretion in the 3/4 nephrectomy and STZ induced diabetic rats groups [49]. In the subsequent mechanism study, XKG was proved to exert renal protective effect in DN through downregulating TGF-β1 expression in rat mesangial cells [50].

2.1.20. Xiexin Decoction (XXD). Xiexin decoction (XXD) is composed of three crude drugs including radix et rhizoma rhei, rhizoma coptidis, and radix *Scutellaria* and has been used for the treatment of DM for at least 1700 years. One study in HFD-fed plus STZ induced rats showed that XXD could attenuate albuminuria and renal pathological changes, reduce AGEs, inhibit RAGE and inflammation factors expression, suppress NF-κB, and downregulate renal TGF-β1. All these data suggested that renal protective potential of XXD was involved in inhibition of inflammation through downregulating NF-κB pathway, reducing renal AGEs and RAGE in diabetic rats [51]. A recent study of XXD components in db/db mice showed that multicomponent herbal therapeutic formulations could be a useful approach for the treatment of DN through reducing the expression of NF-κB and TGF-β1 [52].

2.1.21. Xianzhen Tablet (XZT). Xianzhen tablet (XZT, a Chinese patent compound recipe), is composed of astragali radix, radix *Rehmannia*, fructus ligustri lucidi, *Scutellaria baicalensis* Georgi, rhizoma coptidis, dodder weed, fairy spleen, and *Salvia miltiorrhiza*. XZT was reported to decrease blood glucose and HbA1c in diabetic rats, improve renal function, ameliorate proteinuria, and reduce glomerular extracellular matrix expansion and thickness of basement membrane, which was mediated by the inhibition of AGEs accumulation and RAGE mRNA levels in the kidney cortex of STZ induced diabetic rats [53].

2.1.22. Zhibai Dihuang Pill (ZDP). Zhibai Dihuang Pill (ZDP) is one of the TCM preparations, composed of rhizoma anemarrhenae, cortex phellodendri, radix *Rehmannia* preparata, rhizoma *Dioscorea*, fructus corni, cortex Moutan, rhizoma Alismatis, and *Poria*. ZDP has been revealed to have protective effects in experimental DN animal models and DN patients. In a recent metabonomic analysis of ZDP in the treatment of STZ induced diabetic rats, ZDP could ameliorate DN by intervening in some dominant metabolic pathways such as inhibiting glucose and lipid metabolism and enhancing methylamine metabolism [54].

2.1.23. Zao Huang Mixture (ZHM). Zao Huang Mixture (ZHM) is composed of extracts of *Sargassum* and rhizoma rhei. One study has shown that ZHM could prevent the process of DN by decreasing the expression of TGF-β1 and type IV collagen in HG stimulated human glomerular mesangial cells [55].

2.1.24. Zhenqing Recipe (ZQR). Zhenqing Recipe (ZQR), a Chinese herbal prescription composed of 3 crude drugs: fructus ligustri lucidi, *Eclipta prostrate*, and *Dioscorea opposite*, has been used to improve renal function of DN patients. In the study for the underlying mechanism, ZQR has been shown to inhibit the overexpression of TGF-β1 and FN in the renal cortex of HFD-fed plus STZ induced diabetic rats; its renoprotective effect was mediated by inhibiting SREBP-1c overexpression and its target genes including ACC and FAS [17].

2.1.25. Zishentongluo (ZSTL). Zishentongluo (ZSTL) is composed of eleven Chinese herbs: raw *Astragalus*, *Angelica*, safflower, zedoary turmeric, Dodder, radix *Rehmannia*, dogwood, *Poria*, *Epimedium*, earthworm, and *Schisandra*. ZSTL has been shown to be superior to benazepril in improving the metabolic and renal function in DN patients at early stage partially by modifying ANP, VEGF, and ET-1 expressions [56].

2.2. Monomers/Single TCM in DN. With the development of modernization of TCM preparations and applications in the treatment of DN, pharmacological complexity is difficult to be distinguished for the precise underlying mechanism, and, to avoid the toxicity and side effects, there is an increasing interest of single herbal TCM and/or monomers from herbal TCM in the treatment of DN, and they are more appropriate than TCM preparations to clarify the precise action mechanism on DN. All the single herbal TCM and monomers are listed in Table 2.

2.2.1. Astragalus/Radix Astragali. Astragalus (Huang Qi in Chinese), also named as radix astragali, is a TCM from Mongolian milkvetch or *membranaceus* milkvetch. A meta-analysis comprising 25 studies showed that *Astragalus* injection had more therapeutic effect in DN patients such as decreasing BUN, Scr, and urine protein and improving Ccr and serum albumin level [63], and rebalancing TGF-β/Smad signaling could be a potential mechanism to prevent DN in KK-Ay mice [64]. *Astragalus* may protect diabetic rats kidney mediated by downregulation of Tie-2 [110], and radix astragali was reported to upregulate c-met expression in human kidney fibroblasts to delay the progression of DN [65]. Two major isoflavonoids in radix astragali, calycosin and calycosin-7-O-beta-D-glucoside, could inhibit HG induced mesangial cell early proliferation and AGEs-mediated cell apoptosis, suggesting these two isoflavonoids have therapeutic potential to prevent the progression of DN [111]. A recent review showed that total polysaccharides, flavonoids fractions, saponins, and several isolated compounds have antidiabetic potentials, which throw light upon further investigations that should be conducted on the treatment of DN and relevant underlying mechanism [112]. Astragaloside IV (ASI) in radix astragali is considered to be an active constituents; ASI could inhibit human tubular epithelial cells apoptosis and reduce TGF-β1 expression, suggesting a new

Table 2: Applications of single herbal TCM and/or monomers in DN.

Name	Origins	Methods	Results	Pathways
Astragalus	Radix astragali	*Human study* (DN patients)	Decreasing BUN, Scr, and proteinuria and improving CCr and serum albumin level	[63]
		Type 2 diabetic animal study (KK-Ay mice)	Increasing Smad7 expression, inhibiting TGFβR-1, Smad3, and its phosphorylation expression, and decreasing TGF-β1 mRNA level	Rebalancing TGFβ/Smads signaling [64]
		Cellular study (kidney fibroblast)	Upregulating c-met expression	c-met pathway [65]
BBR	*Coptis chinensis, Hydrastis Canadensis, Berberis aristata, Berberis aquifolium*, and *Arcangelisia flava*	*Type 2 diabetic animal study* (HFD + STZ induced rats)	Suppressing histological and ultrastructural changes in kidney, improving glucose and lipid metabolism disorder, increasing cAMP, downregulating GRK2 and GRK3, and upregulating GRK6	Modulating the expression of GRKs in G protein-AC-cAMP signaling pathway [66]
Curcumin	*Curcuma longa* L. (*CLL*)	*Type 2 diabetic animal study* (db/db mice)	Decreasing albuminuria and attenuating glomerular sclerosis	Inhibiting phosphorylation of STAT3 and degradation of IκB [67]
		Cellular study (mesangial cells)	Reducing AGE-induced oxidative stress and restoring AGE-induced mesangial cell apoptosis; Loganin inhibits FN and IL-6 expression	Reducing AGEs-induced ROS [68]
DMDD	Tuberous roots of *A. carambola* L.	*Type 2 diabetic animal study* (KK-Ay mice)	Decreasing hyperglycemia, renal AGE formation, RAGE, Scr, Ccr, and NF-κB, TGF-β1 and enhancing reduced SOD activities	Decreasing AGEs and TGF-β1 levels [69]
DP	Dragon's blood	*Cellular study* (human mesangial cells)	Preventing renal fibrosis	Inhibiting SGK1 and FN expression [70]
EGB	*Ginkgo biloba* leaves	*Human study* (DN patients)	Decreasing urinary mALB, α1-MG, IgG, TF, RBP, and NAG	Through decreasing sICAM-1 and sVCAM-1 [71, 72]
		Cellular study (mesangial cells)	Suppressing MC hypertrophy and ECM accumulation	Through TGF-β1 and Smads pathway [73]
FA	Seeds and leaves of plants	*Type 1 diabetic animal study* (OLETF rats)	Decreasing blood glucose and urinary ACR, mesangial matrix expansion, and glomerular basement thickness	Through reducing oxidative stress and inflammation [74, 75]
Flos *A. manihot*		*Type 1 diabetic animal study* (STZ induced rats)	Preventing renal damage and podocyte apoptosis	[76]
Genipin	*Gardenia jasminoides*	*Type 1 diabetic animal study* (STZ induced mice) *Cellular study* (mouse podocyte)	Ameliorating body weight loss and urine albumin leakage, attenuating GBM thickness, suppressing upregulation of UCP2, and restoring podocin and WT1 expression	Through suppressing upregulation of mitochondrial UCP2 [77]
HCT	*Houttuynia Cordata* Thunb.	*Type 1 diabetic animal study* (STZ induced rats)	Reducing UAER, Ccr, TGF-β1, and collagen I and increasing BMP-7	Decreasing TGF-β1 and increasing BMP-7 [78]
Icariin	Herba epimedii	*Type 1 diabetic animal study* (STZ induced rats)	Relieving renal damage	Inhibiting TGF-β1 and Col IV expression [79]
LAB	*Salvia miltiorrhiza*	*Type 1 diabetic animal study* (STZ induced rats)	Renal MDA$\downarrow$, microalbuminuria$\downarrow$, mesangial expansion$\downarrow$, and glomerular hypertrophy$\downarrow$	TGF-β1 pathway [80]
		Cellular study (mesangial cells)	TGF-β1 and fibronectin secretion$\downarrow$ and PKC and ROS$\downarrow$	PKC and ROS pathway [80]
		Cellular study (VSMCs)	Inhibiting VSMCs proliferation and migration	Nrf2-ARE-NQO1 [81]

Table 2: Continued.

Name	Origins	Methods	Results	Pathways
LBP	Fruit of goji berry	*Type 1 diabetic animal study* (STZ induced rats)	Increasing antioxidant enzymes and increasing scavenging oxygen radicals	Via decreased ERK 1/2 activation through PKC [82]
LGP	*Averrhoa carambola* L. (Oxalidaceae) root	*Type 1 diabetic animal study* (STZ induced mice)	Decreasing hyperglycemia, NF-κB, caspase-3, caspase-8, caspase-9, and Bax expression; alleviating glomerular hypertrophy and ECM accumulation	[83]
Ligustrazine	Chuangxiong	*Human study* (DN patients)	Reducing BUN, Scr, 24 h urine protein, urine mAlb, and UAER	[84]
MC	Moutan cortex	*Type 2 diabetic animal study* (HFD + STZ induced rats)	Increasing SOD, GSH-PX, and CAT, reducing MDA; decreasing blood glucose, Scr, and urine protein and downregulating TGF-β2; decreasing IL-6 and MCP-1, TGF-β1, ICAM-1, and RAGE	Through attenuating oxidative stress and ameliorating inflammation [85–87]
		Cellular studies (HBZY-1 mesangial cell, rat mesangial cells)	Downregulating FN and collagen IV expression	
Morroniside	*Corni fructus*	*Type 1 diabetic animal study* (STZ induced rats)	Increasing decreased serum ALB, reducing elevated BUN, and slowing down Ccr decrease	Through inhibiting hyperglycemia and oxidative stress [88]
PNS	Radix notoginseng	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing FBG, Ccr, UAlb, and renal index	Through inhibiting VEGF and TGF-β1 and enhancing BMP-7 and Smad7 [89, 90]
Puerarin	*Pueraria candollei*	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing collagen IV; attenuating kidney hypertrophy, mesangial expansion, and proteinuria	Downregulating MMP-9 and eNOS expression [91–93]
Rhein	Rhubarb	*Type 2 diabetic animal study* (db/db mice)	Decreasing UAE and ECM levels, decreasing TGF-β1 and fibronectin deposition, and decreasing hyperlipidemia	Through decreasing lipid levels [94]
		Cellular study (rat renal PETCs)	Inhibiting cell hypertrophy	[95]
R. rosea	*Rhodiola rosea*	*Type 2 diabetic animal study* (HFD + STZ induced rats)	Reducing FBG, TC, TG, Ccr, and 24 h urinary albumin	Through decreasing TGF-β1 expression [96]
RLM	*Rosa laevigata* Michx.	*Type 1 diabetic animal study* (STZ induced rats)	Increasing SOD activity and total antioxidant capacity, decreasing MDA and ROS levels, and inhibiting NF-κB p65 and MCP-1 expression	Through regulating oxidative stress and inflammation [97]
Sequoyitol	*Aristolochia arcuata*, *Amentotaxus yunnanensis*, and *Crossostephium chinense*	*Type 2 diabetic animal study* (HFD + STZ induced rats)	Decreasing FBG, BUN, and Scr levels, increasing insulin and T-AOC levels in rats, and decreasing $P22^{phox}$, $P47^{phox}$, NF-κB, and TGF-β1 expression in vivo and in vitro	Through glucose-lowering effects, antioxidant activity, and regulation of TGF-β1 expression [98]
SF	*Angelica sinensis*, *Lignsticum chuangxiong*, *Cimicifuga heracleifolia*, and other plants	*Human study* (DN patients)	Lowering UAER level and improving renal function	Through decreasing (ET) and inhibiting the combination of ET with its receptor [99]

Table 2: Continued.

Name	Origins	Methods	Results	Pathways
Skimmin	*Hydrangea paniculata*	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing Scr and blood glucose level, alleviating glomerular segmental sclerosis and tubular vacuolar degeneration, and downregulating TGF-β1 and TGF-β1 receptor I expression	Through inhibiting TGF-β1 pathway [100]
SM	*Salvia miltiorrhiza*	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing TGF-β1, CTGF, PAI-1, FN ED-1, collagen IV, and RAGE overexpression and protecting tubular function and structure	Through inhibiting TGF-β1 pathway, oxidative stress, and inflammation [101–103]
TGP	*Paeonia lactiflora* Pall.	*Type 1 diabetic animal study* (STZ induced rats)	Elevating antioxidant enzyme and decreasing p-p38 MAPK and NF-κB	Through inhibiting oxidative stress [104]
		Type 2 diabetic animal study (HFD + STZ induced rats)	Decreasing Scr, BUN, and 24 h UP and improving renal histopathology	Through inhibiting Wnt/beta-catenin signaling pathway [105]
TMP	*Ligusticum chuanxiong*	*Type 1 diabetic animal study* (STZ induced rats)	Improving renal function	Through downregulating VEGF expression [106]
Triptolide	Diterpene purified from TwHF	*Type 2 diabetic animal study* (db/db mice)	Decreasing albuminuria, alleviating glomerular hypertrophy and podocyte injury, and attenuating inflammation and oxidative stress in kidney	Through inhibiting inflammation and dyslipidemia [107]
TwHF		*Human study* (DN patients)	Preventing podocyte injury	Downregulating TGF-β1, OPN, and CTGF [108]
VOMBP	*Magnolia biondii* Pamp.	*Type 1 diabetic animal study* (STZ induced rats)	Decreasing 24 UmAlb, sP-selectin in serum, and P-selectin in renal tissue	Inhibiting P-selectin [109]

treatment for DN probably mediated by the inhibition of p38 MAPK pathway activation and HGF overproduction [113].

2.2.2. Berberine (BBR). Berberine (BBR), an effective compound of herbal TCM, includes *Coptis chinensis*, *Hydrastis Canadensis*, *Berberis aristata*, *Berberis aquifolium*, and *Arcangelisia flava*.

BBR treatment could restore renal functional parameters, improve glucose and lipid metabolism disorders, suppress alterations of histological and ultrastructural changes in kidney, and increase cAMP levels in HFD-fed plus STZ induced diabetic rats, and the renal protective effect is exerted by modulating the G protein-coupled receptor kinases (GRKs) in G protein-AC-cAMP signaling pathway [66]. A previous study showed that BBR-containing TCM could increase glucose uptake and lipid oxidation with insulin sensitivity in Zucker diabetic fatty rats [16].

2.2.3. CLL/Curcumin. Curcuma longa L. (CLL) has been widely used to prevent diabetic vascular complications in recent years. Curcumin and demethoxycurcumin are isolated from CLL and have been shown to potentially protect DN by reducing AGE-induced oxidative stress and restoring AGE-induced mesangial cell apoptosis [68]. In the treatment of DN in db/db mice, curcumin has been shown to decrease albuminuria and attenuate glomerular sclerosis by inhibiting phosphorylation of STAT3 and degradation of IκB [67]. A systemic review and meta-analysis of fourteen randomized controlled trials suggested that curcumin has protective potentials on the kidneys of diabetic rats/mice [114].

2.2.4. 2-Dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione (DMDD). 2-Dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione (DMDD), isolated from the tuberous roots of *A. carambola* L. (Oxalidaceae), has been shown to enhance the reduced SOD activities in the kidney of KK-Ay mice and inhibit the progression of DN through decreasing AGEs and TGF-β1 levels [69].

2.2.5. Dracorhodin Perchlorate (DP). Dracorhodin perchlorate (DP), one of the main compositions of Dragon's blood, has been shown to prevent and retard renal fibrosis of DN partially through inhibiting SGK1 and FN expression in human mesangial cells [70].

2.2.6. EGB. Ginkgo biloba extract (EGB), taken from the leaves of *Ginkgo biloba*, is a mixture containing flavonoid glycosides and has been proven to ameliorate hemodynamics, suppress PAF and ACE activities, scavenge ROS, relax vascular smooth muscles, and suppress AGEs expression. In a previous study on DN patients, EGB treatment has been shown to decrease urinary mALB, α1-MG, IgG, TF, RBP, and NAG in DN patients compared with control group, which suggested that EGB has renoprotective effect on the early DN [71]. The subsequent mechanism study showed that EGB could suppress rat mesangial cells hypertrophy and ECM accumulation through decreasing Smad2/3 and TGF-β1 and increasing Smad7 [73], while in DN patients EGB has been proven to retard early DN development through decreasing serum sICAM-1 and sVCAM-1 levels [72].

2.2.7. Flos Abelmoschus manihot. Flos *Abelmoschus manihot* (Huangshukuihua in Chinese) has been widely used as the neuroprotective drug for cerebral ischemic reperfusion injury. Total flavone glycosides of flos *A. manihot* (TFA) contain 7 identified flavone glycosides. TFA pretreatment has been shown to prevent renal damage and podocyte apoptosis in STZ induced rats [76]. A meta-analysis of 27 randomized controlled trials showed that flos *Abelmoschus manihot* had significant effect on renal function in the treatment of DN deserving further investigation [115].

2.2.8. Genipin. Genipin is a glycone derived from geniposide present in fruit of *Gardenia jasminoides*. Genipin has been proven to ameliorate body weight loss and urine albumin leakage, attenuate GBM thickness, and restore the podocyte expression of podocin and WT1 in diabetic mice; the protective effect of Genipin on DN is probably through suppressing the upregulation of mitochondrial UCP2 in STZ induced diabetic mice kidneys [77].

2.2.9. Houttuynia cordata Thunb. (HCT). Houttuynia cordata Thunb. (HCT, Yu Xing Cao in Chinese), pungent in taste and cool in nature, has been reported to reduce urinary proteins in the patients with nephrotic syndrome; HCT has also been shown to protect diabetic kidney function through decreasing the expression of TGF-β1 and increasing the expression of BMP-7 [78].

2.2.10. Icariin. Icariin is a major constituent of flavonoid extracted from the plant herba epimedii and has been shown to relieve renal damage in STZ induced diabetic rats through inhibiting the expression of TGF-β1 and collagen IV protein [79].

2.2.11. LAB. Lithospermate B (LAB), a tetramer of caffeic acid isolated from *Salvia miltiorrhiza* radix, was identified as antioxidant and PKC inhibitor in the renoprotective effects under diabetic conditions *in vivo* and *in vitro* [80]. In the STZ induced diabetic rats, delayed LAB treatment could inhibit renal MDA, microalbuminuria, mesangial expansion, and glomerular hypertrophy, and in mesangial cells LAB could inhibit HG and H_2O_2 induced TGF-β1 and FN secretion, HG induced intracellular PKC activation, and ROS generation, which suggested that LAB could significantly suppress the progression of diabetic renal injury. A recent study showed that LAB could prevent diabetic atherosclerosis by induction of the Nrf2-ARE-NQO1 pathway to inhibit VSMCs proliferation and migration and vascular damage [81]. All these findings suggested that LAB could be a new therapeutic agent in the treatment of DN. In the subsequent study, *Salvia miltiorrhiza* could protect STZ induced diabetic rats by inhibiting the overexpression of TGF-β1, CTGF, PAI-1, and FN in renal cortex.

2.2.12. LBP. Lycium barbarum polysaccharide (LBP) is extracted from the fruit of goji berry(Solanaceae); LBP4

has been shown to protect STZ induced diabetic kidney function via decreasing the activation of ERK1/2 through the involvement of PKC in mesangial cells [82].

2.2.13. LGP. Lyoniresinol 3 alpha-O-beta-D-glucopyranoside (LGP) is isolated from *Averrhoa carambola* L. (Oxalidaceae) root (ACLR), including two chiral lignin glucosides: LGP1 and LGP2. LGP1 treatment has been shown to decrease hyperglycemia and the expression of related proteins including NF-κB, caspase-3, caspase-8, caspase-9, and Bax in STZ induced diabetic mice. LGP1 also could alleviate glomerular hypertrophy, excessive ECM accumulation, and glomerular and tubular basement membrane thickness. All these data suggested that LGP1 could be a potential therapeutic agent in DN [83].

2.2.14. Ligustrazine. Ligustrazine, a bioactive component of Chuangxiong, has been widely used in the treatment of vascular diseases such as myocardial and cerebral infarction in China. A meta-analysis of 25 studies showed that Ligustrazine has therapeutic effect to improve renal function and reduce urine protein excretion in DN patients [84]. Further studies should be conducted to reveal the underlying mechanism for the treatment on DN.

2.2.15. MC. Moutan cortex (MC), the root bark of *Paeonia suffruticosa*, has been shown to have the protective effect against atherosclerosis and inflammation and inhibitory effect on the production of ROS. MC was reported to increase activity of SOD, GSH-PX, and CAT and reduce MDA *in vitro* or *in vivo*; furthermore, MC could decrease blood glucose, Scr, and urine protein in HFD-fed plus STZ induced diabetic rats, which suggested that MC has renal protective effect in AGEs-induced mesangial cell dysfunction through attenuating oxidative stress pathway [85], while, in AGEs-induced rat mesangial cells, MC could inhibit FN and collagen IV expression in matrix [86]. Apart from the abovementioned evidence of renal protective effect on DN, MC could ameliorate activity on the inflammation via target of RAGE *in vitro* or *in vivo* [87].

2.2.16. Morroniside. Corni fructus, a constituent of HJG, used as a traditional medicine in China and Japan, has been shown to be superior to aminoguanidine treatment in suppressing hyperglycemia, proteinuria, renal AGE formation, and TGF-β1 expression in STZ induced diabetic rats [116]. Morroniside, isolated from corni fructus, could exhibit protective effects against STZ induced renal damage by inhibiting hyperglycemia and oxidative stress [88]. Another study showed that components of corni fructus could play protective effect on early stage of DN in type 2 diabetic rats mediated by the regulation of podocytes. Loganin from corni fructus and its derivatives could inhibit the expression of FN and IL-6 in the HG stimulated mesangial cells, which supported the traditional use of corni fructus in DN and relevant kidney diseases [117].

2.2.17. Panax Notoginoside (PNS). Panax notoginoside (PNS) is extracted from radix notoginseng and has been shown to protect kidney in type 1 diabetic rats at early stage through inhibiting the expression of VEGF protein and enhancing BMP-7 expression in the kidney [89]. Another report showed that the protective effect of PNS in kidney was mediated by inhibiting TGF-β1 expression and enhancing the expression of Smad7 [90]. Ginsenoside Rg1, an active ingredient isolated from PNS, has been shown to improve the renal pathological changes in STZ induced diabetic rats through reducing TGF-β1 expression and inflammatory reaction factors including CRP and TNF-α [118]. Ginsenoside Rg1 also could effectively relieve aldosterone-induced oxidative stress through which it indirectly inhibits aldosterone-induced podocyte autophagy [119].

2.2.18. Puerarin. Puerarin, 7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one-8-b-D-glucopyranosid-e, is one of the major isoflavonoid compounds from the root of *Pueraria candollei* wall of Leguminosae family. A previous study showed that Puerarin could protect DN rats by inhibiting collagen IV expression [91]; further study in STZ induced diabetic rats showed that Puerarin could protect kidney function through downregulating MMP-9 and attenuating eNOS expression [92, 93].

2.2.19. Rehmannia Radix (Di Huang). Rehmannia radix (Di Huang) was mostly mentioned and investigated; it has been proven to reduce hyperglycemia, ameliorate renal dysfunction, prevent senility, and improve hemorheology. In a previous experimental study *Rehmannia* radix has been shown to inhibit the progression of DN [120]. Catalpol is an iridoid glucoside compound mainly present in *Rehmannia* radix and other plants amd has been shown to reduce ECM accumulation by inhibiting the expression of TGF-β1, CTGF, and Ang II in HFD-fed plus STZ induced diabetic rats [121].

2.2.20. Rhein. Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is purified from rhubarb (*Rheum officinale*). Rhein has shown reduction of UAE faster than simvastatin and decrease of ECM levels along with decreased TGF-β1 and FN immunohistochemistry expression in db/db renal tissue, which was supposed via regulation of dyslipidemia [94]. Another study showed that Rhein could inhibit the hypertrophy of rat renal proximal tubular epithelial cells stimulated by HG and Ang II [95].

2.2.21. Rhodiola rosea. Rhodiola rosea (*R. rosea*) is grown at northern latitudes and high altitudes of the world; *Rhodiola rosea* extract has been used to protect kidney function including reducing FBG, TC, TG, Ccr, and 24 h urinary albumin in HFD-fed plus STZ induced diabetic rats through decreasing renal expression of TGF-β1 [96].

2.2.22. Rosa laevigata Michx. (RLM). Rosa laevigata Michx. (RLM), a commonly used TCM for the treatment of urinary tract infection and antioxidative treatment, could play a critical role in the pathogenesis of DN through increasing the activity of SOD and total antioxidant capacity, decreasing MDA and ROS levels, and inhibiting NF-κB p65 and MCP-1 expression following increased IκB protein expression in STZ

induced diabetic rats; all the data suggested that RLM could be a therapeutic potential for DN [97].

2.2.23. Sequoyitol. Sequoyitol is a natural compound present in a lot of plants (e.g., *Aristolochia arcuata*, *Amentotaxus yunnanensis*, and *Crossostephium chinensis*); oral and subcutaneous administrations of sequoyitol could ameliorate hyperglycemia and glucose intolerance in ob/ob mice. Sequoyitol has been shown to ameliorate the progression of DN in HFD-fed plus STZ induced rats through glucose-lowering effects, antioxidant activity, and regulation of TGF-β1 expression [98].

2.2.24. SF/FA. Sodium ferulate (SF), extracted from *Angelica sinensis*, *Lignsticum chuangxiong*, *Cimicifuga heracleifolia*, and other plants, has platelet aggregation inhibitory, antithrombotic, and antioxidant activities in animals and humans. A preliminary study on DN patients showed that SF could lower UAER level and improve renal function through decreasing endothelin (ET) and inhibiting the combination of ET with its receptor [99]. A meta-analysis of 14 randomized controlled trials involving 906 patients showed that SF is superior in reducing UAER, ET, BUN, Scr, and TC and increasing HDL-c without affecting FBG and TG [122]. Ferulic acid (FA) is a phenolic acid extracted from the seeds and leaves of most plants and has antioxidant activities, hypoglycemic and hypolipidemic effects, hypotensive effects, and anti-inflammatory effects. In the FA treated OLETF rats, blood glucose and urinary ACR were decreased significantly; in renal histopathology glomerular basement membrane thickness and mesangial matrix expansion were decreased through reducing oxidative stress and inflammation [74, 75].

2.2.25. Skimmin. Skimmin, a major active component from *Hydrangea paniculata*, has been reported to decrease Scr and blood glucose level and alleviate glomerular segmental sclerosis and incidence of tubular vacuolar degeneration by downregulating the TGF-β1 and TGF-β1 receptor I expression in STZ induced diabetic rats [100].

2.2.26. SM. Salvia miltiorrhiza (SM, commonly known as Danshen in Chinese) has been shown to have the anti-inflammatory, antioxidative, and organ protective effects. A previous study showed that SM could protect STZ induced diabetic rats from DN by suppressing the overexpression of TGF-β1, CTGF, PAI-1, and FN in renal cortex [101]. Another study showed that SM could ameliorate TGF-β1 levels in serum and kidney and reduce the levels of collagen IV ED-1 and RAGE in the diabetic kidney [102]. Danshen injection, the aqueous extracts of SM, could protect diabetic rats associated with preservation of tubular function and structure from hyperglycemia induced oxidative stress, advanced glycation stress, and megalin expression deletion [103].

2.2.27. TGP. Total glucosides of paeony (TGP), extracted from the root of *Paeonia lactiflora* Pall., have been shown to have the therapeutic effect in the experimental DN. TGP treatment in the STZ induced diabetic rats could prevent diabetic renal damage against oxidative stress through decreasing upregulated p-p38 MAPK and NF-κB P65 expressions [104]. And, in the HFD-fed plus STZ induced rats, TGP could improve kidney damage and delay the development of DN by inhibiting Wnt/beta-catenin signaling pathway [105].

2.2.28. TMP. Tetramethylpyrazine (TMP) is isolated from *Ligusticum chuanxiong* and has been used in the treatment of stroke and cardiovascular diseases. TMP was reported to reduce diabetic kidney damage partially by downregulating the expression of VEGF in the kidney [106].

2.2.29. Triptolide/GTW/TwHF. Triptolide, active diterpene purified from *Tripterygium wilfordii* Hook. F. (TwHF), has been reported to have anti-inflammatory, antioxidative, immunosuppressive, and podocyte-protective effects. A recent study showed that triptolide could attenuate albuminuria in db/db diabetic mice accompanied with alleviated glomerular hypertrophy and podocyte injury, while inflammation and dyslipidemia were also attenuated [107]. Triptolide is one of the major active components of multiglycoside of TwHF (GTW), and GTW has been applied extensively for the treatment of CKD in China as an anti-inflammatory agent. GTW could prevent glomerular lesion in STZ induced diabetic model through decreasing urine albumin and ameliorating glomerular sclerosis [123]. A recent study showed that TwHF could prevent podocyte injury of DN patients, which may be partly mediated by downregulating the expression of OPN, CTGF, and TGF-β1 [108].

2.2.30. Volatile Oil of Magnolia biondii Pamp. (VOMBP). Volatile oil of *Magnolia biondii* Pamp. (VOMBP), extracted from herbal TCM *Magnolia biondii* Pamp., has been reported to protect the kidney in STZ induced diabetic rats by inhibiting the expression of P-selectin in serum and renal tissue [109].

2.3. TCMs Combined Therapy with Western Medicines in DN. Apart from the TCM preparations and single TCM applications in DN, TCMs combined with western medicines have been indicated. Mostly used western medicines were ACEI/ARBs, and combination styles included Tangshenling (TSL) with telmisartan [124] in diabetic patients or TSL with benazepril in STZ induced rats [125], triptolide with benazepril in DN patients [126], Bailing Capsule (BC) and benazepril in DN patients [127], and safflower yellow powder injection with benazepril in DN patients [128]. Another report is about Tangshenqing (TSQ) combined with alprostadil in the treatment of DN patients [129]. All data suggested that effects of TCMs combined therapy with western medicines were superior to western medicines treatment alone.

3. Conclusions and Perspectives

Although there are almost no side effects mentioned in numerous scientific reports, a lot of scientific researches indicate that herbal TCM preparations have renal protective

effects on DN according to respective factors, complexity, and variability of TCM preparations still presenting challenges for clinicians seeking scientific evidence to support TCM application in drug discovery. In order to avoid the toxicity and side effects of TCM formulas, there is increasing interest in studying single herbal TCM especially monomers from single herbal TCM on DN. In this review, we found that monomers such as Berberine, curcumin, Ginsenoside Rg1, Puerain, Rhein, and Ferulic acid have specific protective effect on DN. To translate the therapeutic potentials for DN into reality, placebo-controlled and randomized controlled clinical trials of single herbal TCM and/or monomers from herbal TCM are essential in the future, and prompt meta-analysis is an effective alternative.

Conflict of Interests

The authors have no conflict of interests to declare.

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