

The Role of Podocytes and Podocyte-Associated Biomarkers in Diagnosis and Treatment of Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is an important public health problem. Podocyte injury is a central event in the mechanism of DKD development. Podocytes are terminally differentiated, highly specialized glomerular visceral epithelial cells critical for the maintenance of the glomerular filtration barrier. Although potential mechanisms by which diabetic milieu contributes to irreversible loss of podocytes have been described, identification of markers that prognosticate either the development of DKD or the progression to end-stage kidney disease (ESKD) have only recently made it to the forefront. Currently, the most common marker of early DKD is microalbuminuria; however, this marker has significant limitations: not all diabetic patients with microalbuminuria will progress to ESKD and as many as 30% of patients with DKD have normal urine albumin levels. Several novel biomarkers indicating glomerular or tubular damage precede microalbuminuria, suggesting that the latter develops when significant kidney injury has already occurred. Because podocyte injury plays a key role in DKD pathogenesis, identification of markers of early podocyte injury or loss may play an important role in the early diagnosis of DKD. Such biomarkers in the urine include podocyte-released microparticles as well as expression of podocyte-specific markers. Here, we review the mechanisms by which podocyte injury contributes to DKD as well as key markers that have been recently implicated in the development and/or progression of DKD and might serve to identify individuals that require earlier preventative care and treatment in order to slow the progression to ESKD.

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1. An Overview of Diabetic Kidney Disease

Diabetic kidney disease (DKD) is a microvascular complication of diabetes that leads to more than 40% of new end-stage kidney disease (ESKD) cases; it is the most common cause of kidney failure in the United States [1]. A total of 30% to 40% of patients with diabetes

Abbreviations: CD2AP, CD2-associated protein; DKD, diabetic kidney disease; ESKD, end-stage kidney disease; GFR, glomerular filtration rate; MCP-1, monocyte chemoattractant protein 1; ROCK1, Rho-associated coiled coil-containing protein kinase 1; SD, slit-diaphragm; VEGF-A, vascular endothelial growth factor-A

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mellitus develop DKD, with the majority of the cases attributable to type 2 diabetes mellitus, the more prevalent type of diabetes [2].

DKD is a glomerular disease, damaging the glomerular filtration barrier, a tripartite system consisting of fenestrated endothelial cells, the glomerular basement membrane, and podocytes. This barrier is critical to the selective filtration of water and solutes; it impedes the passage of macromolecules, such as albumin. Furthermore, podocyte injury with eventual podocyte loss is a critical event in the development and eventual progression of DKD. The hallmark histological changes associated with DKD comprise basement membrane thickening, podocyte loss, and mesangial expansion, with eventual nodular sclerosis in the later stages [3, 4]. These late stages are often associated with arterial hyalinosis and tubulointerstitial fibrosis [4]. The changes also cause microalbuminuria that can eventually progress to macroalbuminuria. Twelve percent of patients with diabetes mellitus type 1 develop macroalbuminuria after an average diabetes duration of 29 years [5]. Currently, albuminuria is the most widely used earliest clinical marker of DKD. Albuminuria is independently associated with increased cardiovascular morbidity and mortality and may be the best predictor of a future decline in glomerular filtration rate (GFR) [6]. However, approximately 30% individuals with DKD do not develop albuminuria [7].

The risk of developing DKD strongly correlates with the duration of diabetes [8]. Hyperglycemia generates reactive oxygen radicals and advanced glycation end-products, which bind to advanced glycation end-product receptors, triggering downstream signaling that facilitates production of reactive oxygen species, activates inflammatory cells, increases synthesis of angiotensin II, and causes a release of growth factors. Increased angiotensin II results in proteinuria and stimulates vascular endothelial growth factor-A (VEGF-A) and TGF- β . The currently available forms of medical therapy capable of reducing proteinuria and delaying the progression of DKD are angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and sodium-glucose cotransporter-2 inhibitors [9–12].

VEGF-A is necessary for the normal development of glomerular capillaries and for maintenance of podocytes via Akt signaling. In the early stages of DKD, hyperglycemia inhibits nitric oxide production by endothelial cells; this inhibition stimulates podocytes' excessive synthesis of VEGF-A, which induces growth and proliferation of mesangial and endothelial cells. In later stages of diabetic nephropathy, podocytes are lost; as a result, VEGF-A synthesis is reduced negatively affecting podocyte health, endothelial fenestration, and glomerular basement membrane composition [13, 14].

TGF- β is a hypertrophic pro-sclerotic cytokine that triggers Smad signaling and contributes to renal hypertrophy, GBM thickening, accumulation of mesangial extracellular matrix, and podocyte detachment. TGF- β stimulates synthesis of VEGF-A triggering a vicious cycle of glomerular damage [15–17].

The processes described here eventually lead to a decline in GFR and eventual ESKD [18].

2. Podocyte Structure and Function

Podocytes are terminally differentiated, highly specialized glomerular visceral epithelial cells that consist of a cell body primary processes and branching foot processes. Podocytes are characterized by a high rate of vesicular traffic as evidenced by multiple coated vesicles and coated pits along the basolateral domain of these cells. Podocytes have high capacity for protein synthesis and posttranslational modifications because of a well-developed endoplasmic reticulum and a large Golgi apparatus [19].

Podocytes wrap around glomerular capillaries; the cells' foot processes interdigitate with those of neighboring podocytes creating 40-nm-wide filtration slits, evenly spaced areas covered by slit-diaphragm (SD) proteins that facilitate podocyte-to-podocyte contact. A multiprotein complex, the SD plays an important role in blood filtration and podocyte signal transduction. Proteins necessary for a proper function of the SD include nephrin, podocalyxin, P-cadherin, NEPH1, NEPH2, podocin, CD2-associated protein (CD2AP), and

FAT1. Podocytes participate in regulating GFR: when podocytes contract, they close filtration slits and, thus, reduce the surface area available for filtration [19–21].

Podocytes' integrity is crucial for maintenance and function of an intact glomerular filtration barrier: they are the primary source of GBM components laminin beta 2 and collagen IV; they initiate fenestrae formation in endothelial cells, endocytose filtration retentae, including albumin and immunoglobulins; and they secrete vascular endothelial growth factor VEGF-A and angiopoietin-1, molecules required for survival of endothelial cells. Although terminally differentiated, the cells are highly dynamic, interacting with the glomerular basement membrane and communicating through signaling at the slit diaphragm [19–21].

3. The Role of the Podocyte in the Pathogenesis of Diabetic Kidney Disease

Podocyte loss is a key factor contributing to DKD progression [22–24]. Because podocytes are terminally differentiated cells, their loss is an irreversible event that leads to a decline in the function of the glomerular filtration barrier [21, 25].

A potential mechanism by which the diabetic milieu contributes to podocyte injury is a cytoskeletal rearrangement of podocytes reflected by flattening, widening, and retraction of foot processes, a phenomenon called “effacement.” Foot process effacement signifies podocyte injury and weakens the integrity of the glomerular filter barrier, resulting in albuminuria [16, 25].

An important component of effacement mechanism is dysregulation of nephrin production. Nephrin is an essential transmembrane protein in the slit diaphragm complex involved in podocyte survival [26–29]. Nephrin's intracellular domain functions as an intracellular signaling scaffold that recruits proteins, such as phosphoinositide-3-kinase, the Src family kinase Fyn, and phospholipase C gamma 1 to its tyrosine phosphorylated cytoplasmic tail. Binding of these proteins facilitates nephrin's regulation of the podocyte cytoskeleton through Nck adaptor proteins [30, 31]. The expression of nephrin and its mRNA are decreased in diabetic nephropathy; the decrease results in aberrant rearrangement of actin and breakdown of the slit diaphragm and leads to foot process effacement [32]. Nephrin is also important in regulating podocyte insulin sensitivity; its cytoplasmic domain enables the docking of glucose transporters GLUT1 and GLUT4 with vesicle-associated membrane protein-2, thus facilitating insulin signaling [33, 34].

Another important component of podocyte foot process effacement is dysregulation of the RHO-family of small GTPases, RhoA, Cdc-42, and Rac1 [35]. These molecules are key regulators of actin cytoskeleton remodeling, functioning as molecular switches: they toggle between active and inactive states to coordinate signaling and either promote or inhibit cell motility [35, 36]. Diabetic milieu have been shown to stimulate Rho-GTPase activity, resulting in podocyte actin remodeling and proteinuria. Mutations in specific guanine exchange factors and GTPase activating proteins of the Rho-family GTPases can result in cytoskeletal rearrangement and foot process effacement [35–38].

Podocytes adhere to the glomerular basement membrane via alpha3beta1 integrin and dystroglycans. The expression of alpha3beta1 has been shown to be decreased in patients with diabetes and in rats with streptozotocin-induced diabetes, thereby contributing to detachment of podocytes from the GBM [39, 40]. However, some studies showed an increased expression of integrin beta1 subunit and its mRNA in podocytes cultured in high extracellular glucose and exposed to angiotensin II. These contradictory data may be explained by a hypothesis that after initial podocyte loss, the remaining cells exhibit increased adhesion [41].

Other potential mechanisms by which podocyte damage occurs in diabetes include increased oxidative stress, dysregulated TGF- β signaling, altered autophagy, and activation of pro-inflammatory pathways [41]. For instance, podocyte damage under high-glucose conditions is associated with the release of mitochondrial and plasma membrane reactive oxygen species that trigger p38MAPK and NADPH oxidase signaling pathways [42, 43].

Increased production of ROS in the setting of hyperglycemia causes mitochondrial fragmentation and links several important mechanisms underlying development of DKD and other microvascular complications of diabetes. Mitochondrial fission is triggered by Rho-associated coiled coil-containing protein kinase 1 (ROCK1), a downstream effector of RhoA, which recruits dynamin-related protein-1 to the mitochondria and phosphorylates dynamin-related protein-1 at serine 600 residue. It is the latter action of ROCK1 that triggers mitochondrial fission [44–46].

The TGF- β pathway also plays a central role in podocyte apoptosis, both directly and indirectly. In DKD, upregulated TGF- β activates caspase3 via p38MAPK and Smad7; TGF- β also induces Notch and Wnt/beta-catenin signaling, increasing the expression of downstream target genes, including Snail 1, which suppresses nephrin expression.[47, 48] Although upregulated TGF- β signaling could contribute to antiapoptotic signaling, the overall effect of TGF- β upregulation on podocytes has been associated with increased apoptosis [41].

In DKD, podocytes also exhibit decreased flux of autophagy, a process essential for protein and organelle turnover [49, 50]. Decreased utilization of the autophagy-lysosome pathway shifts the degradation pathways toward the ubiquitin-proteasome system; however, the latter is not able to compensate, leading to the accumulation of dysfunctional proteins, which triggers proapoptotic pathways, resulting in eventual podocyte death [49, 50].

Inflammation plays a role in podocyte death as well. Patients with diabetes have increased levels of caspase 1, an inflammation marker that cleaves interleukin-1 β , causing inflammatory cell death called pyroptosis [51, 52]. Hyperglycemia contributes to activation of nucleotide-binding domain and leucine-rich repeat pyrin 3 domain inflammasome, which facilitates DKD development [51, 52]. Neutralization of interleukin-1 β in diabetic mice has been demonstrated to attenuate pyroptosis and ameliorate DKD [51, 52].

In DKD, the surviving intact podocytes undergo hypertrophy to cover the newly denuded GBM from lost podocytes [53]. The molecular event responsible for this hypertrophy is activation of mammalian target of rapamycin signaling, a process responsible for cellular development and regeneration [54, 55]. In patients with DKD and in db/db mice, increased mammalian target of rapamycin signaling is associated with autophagy and Notch reactivation [54, 55]. Activated Notch signaling is critical in the developing kidney with marked suppression in healthy adult kidneys, but can be reactivated in pathological conditions associated with proteinuria [56, 57].

Increased expression of Notch has been shown both sufficient and necessary to induce albuminuria and glomerulosclerosis via TGF- β activation [57]. Notch and TGF- β form a positive feedback loop where TGF- β transcriptionally upregulates the Notch ligand Jagged 1 and Notch activation enhances TGF- β expression [57]. Interestingly, VEGF-A also regulates Notch expression and activity via a positive feedback loop [58].

Increased expression of Wnt/beta-catenin target genes has been demonstrated in both patients with DKD and in murine models of diabetes. At the same time, increased Wnt/beta-catenin activity has been shown to be associated with antiapoptotic and dedifferentiation processes in the podocyte [59]. The latter process is also facilitated by Ctnnb1 gene; podocyte-specific Ctnnb1 knockout mice had increased podocyte differentiation markers WT1, nephrin, podocalyxin, and synaptopodin [59]. Reemergence of developmental genes in terminally differentiated cells like podocytes is an adaptive reaction to cell injury and death; however, expression of Ctnnb1 and Notch inhibits terminal differentiation of cells, thus contributing to dedifferentiation [59].

Another molecule associated with podocytes' role in DKD pathogenesis is KLF6. A knock-down of KLF6 in podocytes increases the susceptibility to streptozotocin-induced DKD in the resistant C57BL/6 mouse strain; the knockdown reduces synthesis of cytochrome c oxidase 2, leading to an increased mitochondrial injury and activation of the intrinsic apoptotic pathway in the setting of diabetes. These findings are consistent with the observation of a significant reduction in glomerular and podocyte-specific expression of KLF6 in kidney

biopsies of patients with progressing DKD. On the other hand, an overexpression of KLF6 leads to a significant attenuation of mitochondrial injury and apoptosis in cultured human podocytes in the setting of hyperglycemia. Thus, KLF6 plays a critical role in preventing mitochondrial injury and apoptosis in the setting of diabetes [46].

The mechanism of podocyte damage in DKD may have an immunological component. Single-cell RNA profiling of glomerular cells (the latter include podocytes, parietal epithelial cells, fenestrated endothelial cells, and mesangial cells) with subsequent cluster analysis showed that the number of immune cells, predominantly macrophages, was significantly higher in diabetic glomeruli compared with control glomeruli [60].

Previous studies demonstrate that the threshold for development of glomerulosclerosis occurs when approximately more than 20% of podocytes are lost, despite the removal of triggering factors contributing to podocyte injury [61]. Podocytes have been shown to transmit injury not only to other glomerular cells, but also to other podocytes [61]. Severe podocyte damage leads to loss of cell-to-cell communication pathways as exemplified by VEGF-A, a factor synthesized by podocytes, but playing a critical role in maintaining endothelial cells [61]. Because the loss of podocytes is irreversible, therapeutic agents targeting early stages of podocyte injury could play an important role in preventing decline of renal function in patients with diabetes [41].

4. The Role of Podocytes and Podocyte-Specific Molecules as Biomarkers of Diabetic Kidney Disease

Currently, the most commonly used marker for early detection of DKD is microalbuminuria; however, this marker has significant limitations: on the one hand, not all diabetic patients with microalbuminuria will progress to ESKD [62]; on the other, as many as 30% of patients with DKD have normal urine albumin levels [63]. Several novel biomarkers indicating glomerular damage precede microalbuminuria, suggesting that the latter develops when significant renal injury has already occurred [64]. Finding new biomarkers that are more sensitive than microalbuminuria and that identify DKD earlier is the goal of many research studies. Because podocyte injury plays a key role in DKD pathogenesis, podocyte-associated biomarkers may play an important role in the early diagnosis of kidney damage in the setting of diabetes.

Urine podocytes and podocyte-specific proteins can serve as urinary markers for early diagnosis of DKD [65, 66]. Podocytes can be detected in urine of diabetic patients with both micro- and macroalbuminuria [67]. Nephritin is present in urine of all diabetic patients with micro- and macroalbuminuria and of 54% of patients with normoalbuminuria; nephritinuria correlates positively with albuminuria. Thus, nephritin can serve as a biomarker of early DKD, although correlating nephritinuria with clinical data in a cross-sectional study does not clarify whether nephritinuria plays an etiological role in DKD or if early nephritinuria can predict DKD development consistently [68]. Nephritin is a 180-kDa transmembrane protein associated with congenital nephrotic syndrome of the Finnish type, an autosomal recessive disorder leading to massive proteinuria and death in infancy. Changes in nephritin excretion have been linked to podocyte injury [69]. Animal models of type 1 diabetes mellitus and of DKD in either streptozotocin-treated rats or Akita mice (the latter have a point mutation in *Insulin2* gene) demonstrated that the peak of nephritinuria preceded changes in urine albumin levels [70, 71]. Analysis of nephritin levels in urine of patients with DKD either by measuring mRNA by RT-PCR or by measuring protein levels by Western blot or ELISA corroborates the data obtained in animal models, shows that nephritinuria is more severe in patients with DKD versus controls and demonstrates a positive correlation of urine nephritin levels with the urine albumin/creatinine ratio and with estimated GFR [72, 73]. Furthermore, Wada et al. suggested that the urinary nephritin-to-creatinine ratio can serve as a reliable marker for predicting the effectiveness of DKD treatment [74].

Another potential podocyte-associated biomarker of DKD is podocalyxin, a highly electronegative sialoglycoprotein and the main podocyte surface antigen, which prevents the podocyte foot processes from collapsing [75]. Urinary podocalyxin level is elevated in 53.8% of diabetic patients with normoalbuminuria, 64.7% of diabetic patients with microalbuminuria, and in 66.7% of diabetic patients with macroalbuminuria [76]. Therefore, podocalyxin may play a useful role as a biomarker of early podocyte injury in DKD.

Monocyte chemoattractant protein 1 (MCP-1) is a cytokine secreted by podocytes as well as by cortical tubular epithelial cells and mononuclear leukocytes; synthesis of MCP-1 is induced by activation of pro-inflammatory nuclear factor-kappa B signaling [77]. MCP-1 has been implicated in renal inflammation, glomerular injury, tubular atrophy, and fibrosis [78] and might be a reliable early biomarker of DKD [79]. Urinary MCP-1 levels correlate with development of DKD in normotensive normoalbuminuric patients with type 1 diabetes before the onset of clinical signs of DKD [80]. High urinary levels of MCP-1 were also found in patients with type 2 diabetes; these levels correlate with the degree of albuminuria [81]. Therefore, MCP-1 may both serve as a biomarker of early DKD and used to assess the degree of renal injury.

Urinary mRNA profiles of podocalyxin, synaptopodin, CD2AP, alpha-actinin-4, and podocin increase in parallel with the progression of DKD, reflecting the severity of albuminuria and renal damage [82]. Synaptopodin plays a critical role in the development and maintenance of the podocyte contractile apparatus by preventing albuminuria through disruption of Cdc42:IRSp53:Mena signaling complex in podocytes [83]. CD2AP is an adaptor protein that binds to nephrin and podocin, anchoring these slit diaphragm proteins to actin filaments of podocyte cytoskeleton and participating in intracellular and extracellular signaling. Podocyte CD2AP is downregulated in diabetic conditions via activation of PI3-K/Act signaling [84]. Alpha-actinin-4 is required for podocyte adhesion; mutations in ACTN4 causes nephrotic syndrome [85]. Podocin participates in the assembly of tight junctions between podocyte foot processes [86]. Quantification of alpha-actinin-4 and podocin in urine may be used to gauge the progression of kidney disease in diabetes [82].

A study performed by Niewczas et al. demonstrated that TNF receptors 1 and 2 are very strong predictors of progression to ESKD in type 2 diabetes patients with and without proteinuria. The association of ESKD with TNFR1 is stronger than that with TNFR2. The cumulative incidence of ESKD for patients in the highest TNFR1 quartile was 54% after 12 years but only 3% for the other quartiles. Plasma TNFR1 levels were able to predict the ESKD risk even after adjustment for clinical covariates such as albuminuria and was better at predicting ESKD than all other clinical variables tested in the study [87].

Niewczas et al. also identified a kidney risk inflammatory signature, consisting of 17 proteins from a systemic, nonrenal source, rich in TNF-receptor superfamily members and correlating with a 10-year risk of end-stage renal disease. Kidney risk inflammatory signature proteins were shown to contribute to the inflammatory process underlying ESKD development in both types of diabetes; they may serve as both therapeutic targets and biomarkers of DKD [88].

Using three different murine type 1 diabetes models (OVE26, STZ-treated, and Akita) and type 2 diabetes db/db mice, Burger et al. demonstrated that podocytes release microparticles when subjected to high glucose conditions or stretching during the earliest stages of DKD; the microparticle release precede changes in urine albumin levels [89]. Microparticles are 0.1- to 1-micron extracellular vesicles that exhibit phosphatidylserine at the surface; these vesicles are released from cell surface in the setting of cellular stress or injury. Thus, urinary podocyte-derived microparticles may serve as early markers of glomerular injury in DKD [89].

Podocyte-associated biomarkers discussed here are summarized in [Table 1](#).

Table 1. Podocyte-associated biomarkers

| Name of the Biomarker | Structural Characteristics of Protein or Organelle | Clinical Significance of Protein or Organelle |
|--|--|--|
| Nephrin (protein or mRNA) | A 180-kDa transmembrane protein | Associated with congenital nephrotic syndrome of the Finnish type (NPHS1). Linked to podocyte injury. Peak nephrinuria precedes albuminuria in animal models |
| Podocalyxin (protein or mRNA) | A highly electronegative sialoglycoprotein | The main podocyte surface antigen; prevents podocyte foot processes from collapsing. Elevated in 53.8% of diabetic patients with normoalbuminuria, 64.7% of diabetic patients with microalbuminuria, and 66.7% of diabetic patients with macroalbuminuria |
| Monocyte chemoattractant protein 1 (MCP-1) | A 13-kDa monomeric polypeptide | A cytokine synthesized via NF-kappa B and secreted by podocytes, cortical tubular epithelial cells and mononuclear leukocytes. Implicated in renal inflammation, glomerular injury, tubular atrophy, and fibrosis. Urinary MCP-1 levels correlate with diabetic nephropathy (DN) in normotensive normoalbuminuric patients with type 1 DM before onset of clinical signs of DN. MCP-1 levels are elevated in DM type 2 and correlate with urine albumin levels |
| Synaptopodin mRNA | A 100-kDa prolin-rich, actin-associated protein | Plays a critical role in the development and maintenance of the podocyte contractile apparatus. Protects against proteinuria by disrupting Cdc42:IRSp53:Mena signaling complexes in podocytes. |
| CD2 associated protein (CD2AP) mRNA | An 80-kDa protein | An adaptor protein; anchors nephrin and podocin (slit diaphragm proteins) to actin filaments of podocyte cytoskeleton. Participates in inward and outward signaling. Downregulated in diabetes via PI3-K/Act signaling. |
| Alpha-actinine-4 mRNA | A 100-kDa protein, belongs to spectrin gene superfamily | Required for podocyte adhesion. Participates in the assembly of tight junctions between podocyte foot processes. Mutations in ACTN4 (the protein's gene) cause an autosomal dominant human kidney disease. |
| Microparticles | 0.1–1 micron extracellular vesicles exhibiting phosphatidylserine at the surface | Released from cell surface in the setting of cellular stress or injury; may serve as early markers of glomerular injury in DN |

5. Conclusion

Podocyte injury is a key event in the pathogenesis of DKD. Podocytes and podocyte-associated molecules have a potential to serve as biomarkers facilitating an earlier diagnosis of kidney damage in diabetes. Understanding the mechanisms of podocyte injury may lead to more effective treatments of DKD and a lower rate of progression to ESKD.

6. Literature Search

To prepare this manuscript, we performed a comprehensive literature search in the common scientific databases for peer-reviewed journals, including PubMed and Cochrane Library. In our search, we used the following key words alone or in combination: *podocytes*, *diabetes*, *diabetic kidney disease*, *biomarkers*, *biological markers* and selected articles we deemed most pertinent to the topic addressed in the manuscript.

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