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# Proteinuria in diabetic kidney disease: A mechanistic viewpoint

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**Proteinuria is the hallmark of diabetic kidney disease (DKD) and is an independent risk factor for both renal disease progression, and cardiovascular disease. Although the characteristic pathological changes in DKD include thickening of the glomerular basement membrane and mesangial expansion, these changes *per se* do not readily explain how patients develop proteinuria. Recent advances in podocyte and glomerular endothelial cell biology have shifted our focus to also include these cells of the glomerular filtration barrier in the development of proteinuria in DKD. This review describes the pathophysiological mechanisms at a cellular level which explain why patients with DKD develop proteinuria.**

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KEYWORDS: podocyte; mesangial cell; glomerular endothelial cell; diabetic nephropathy; albuminuria; proteinuria

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Diabetic kidney disease (DKD) is the leading cause of chronic and end-stage kidney disease, and is epidemic worldwide. The clinical signature of DKD is proteinuria, which is a marker of disease severity and is used clinically to guide our therapies. It is also an independent risk factor for cardiovascular disease.[1,2] Proteinuria is considered to play a central role in the pathogenesis of progressive renal dysfunction. Mechanisms may include enhanced tubular cell uptake of protein leading to complement activation and tubulointerstitial inflammation and increased filtration of pro-oxidant heme proteins, fibrogenic growth factors, and inflammatory cytokines (reviewed in Abbate *et al.*).[3] However, it should be recognized that progressive renal impairment has recently been described in patients with diabetes in the absence of proteinuria, despite having the classic histological features of DKD.[4] Additionally, studies have detected a linear decline in renal function prior to the development of overt proteinuria, questioning whether the loss of glomerular filtration rate (GFR) is etiologically linked to proteinuria or whether the two may occur in parallel.[5]

There has been an exciting increase in our understanding of the mechanisms underlying proteinuria at a molecular and cellular level. Although the exact chronological sequence of events leading to the functional and histological characteristics of DKD is not well defined, our review is based on a model in which proteinuria is a double-edged sword: it serves as a clinical indicator/marker of injury to the kidney, yet also plays an integral role in the pathogenesis of DKD (see Figure 1). In this review, we will not discuss the clinical significance of proteinuria, the risk factors leading to DKD, nor the treatment of proteinuria and disease progression, as these have been keenly discussed in excellent reviews elsewhere.[3,6,7] For the most part, we will also not discuss the pathobiology leading to the individual cellular changes, which are highlighted in Table 1 and reviewed elsewhere.[8,9] The intent of this review is to rather focus comprehensively on the underlying mechanisms of proteinuria in DKD and to highlight the major advances in this area.

## Proteinuria and albuminuria, not microalbuminuria

Although approximately 20% (180 l) of renal plasma flow is filtered at the glomerulus daily, only small amounts of protein are detected in normal urine (40–80 mg day$^{-1}$). Transient increases in proteinuria may occur with fever,

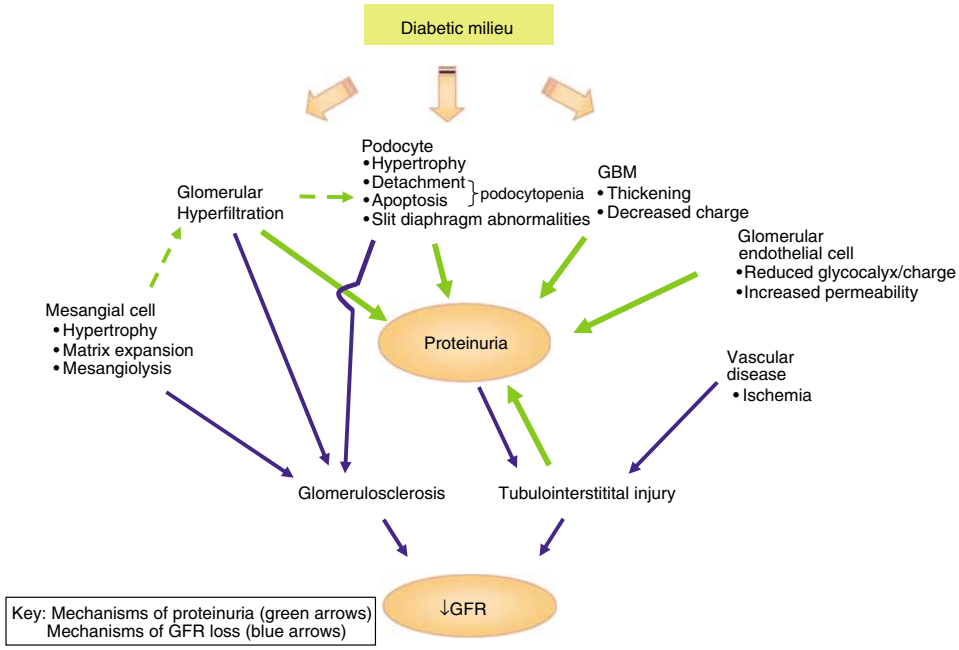


**Figure 1 | Proposed schema unifying the mechanisms of proteinuria and decrease in GFR in DKD.** This schema summarizes events leading to albuminuria/proteinuria (represented by the green arrows) and reduced GFR (represented by the purple arrows) in patients with DKD. The diabetic milieu has effects on all cell types within the kidney (represented by the thick arrows) and these contribute either primarily or secondarily to the development of albuminuria/proteinuria and reduced GFR. At the level of the glomerulus, both hemodynamic effects and injury to the individual components of the glomerular filtration barrier (podocyte, GBM, and glomerular endothelial cell) primarily lead to proteinuria (green arrows). In addition, tubulointerstitial injury may diminish tubular protein reuptake. Mesangial cell injury likely contributes secondarily to proteinuria by (i) mesangial expansion causing a loss of glomerular filtration surface area leading to glomerular hyperfiltration (dashed green arrows) or (ii) by mesangiolysis leading to structural changes in the capillary loops. Proteinuria itself may result in a decrease in GFR by causing tubulointerstitial injury.

**Table 1 | Mechanisms of proteinuria in DKD**

| Site of injury | Effect | Underlying mechanisms |
|---|---|---|
| Glomerular hemodynamics | Glomerular hyperfiltration | Afferent arteriole vasodilatation<br>Efferent arteriole vasoconstriction<br>↑glomerular capillary pressure |
| *Glomerular endothelial cell* | Endothelial cell injury | Hyperglycemia, AGE, ROS |
| | Diminished endothelial glycocalyx | Endothelial cell injury or enzymatic cleavage |
| | Altered VEGF signaling | Podocyte injury or loss |
| *GBM* | Irregular thickening | ↓production and/or ↑degradation of extracellular matrix proteins |
| | Decreased negative charge | ↓ production and/or ↑ degradation of HSPG |
| *Podocyte* | Podocytopenia | Detachment<br>Apoptosis<br>Lack of proliferation |
| | Loss of slit diaphragm integrity | Decrease or changes in subcellular localization of nephrin |
| | Foot process widening and effacement | Disrupted actin cytoskeleton<br>Loss of slit diaphragm integrity<br>Impaired podocyte GBM interaction |
| | Loss negative charge | ↓Podocalyxin |
| Proximal tubule | Decreased protein reabsorption | Tubular injury and interstitial fibrosis |

AGE, advanced glycosylation end products; DKD, diabetic kidney disease; GBM, glomerular basement membrane; HSPG, heparan sulfate proteoglycan; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

marked exercise or exacerbations of congestive heart failure, or hypertension. Urine protein typically comprises albumin (30–40%), Tamm–Horsfall protein (50%), immunoglobulins (5–10%), and light chains (5%). Any protein filtered at the glomerulus is typically taken up by, and degraded in, proximal tubular cells, then reabsorbed into peritubular capillaries. However, peptide fragments are present in human urine (2–3 g day$^{-1}$), that are not detected by standard protein assays.[10,11] Patterns of urine protein fragmentation have been investigated and may correlate with specific glomerular diseases.[12]

The term 'microalbuminuria' is widely used to denote low-grade albuminuria (30–300 mg day$^{-1}$) and identifies those at risk of DKD, and those at risk of cardiovascular

disease in both diabetic and nondiabetic populations. However, it is now well recognized that, even at 'sub-microalbuminuric' levels (albuminuria, 2–30 mg day$^{-1}$), the risk of cardiovascular disease increases, correlating with the degree of albumin excretion.[1,2,13] In view of this, we prefer to consider urinary albumin excretion as a continuous variable and do not consider a specific cutoff in quantity at which the mechanisms of albuminuria may differ. Hence, when we use the term proteinuria, we define this as any amount of protein in the urine (that is, micro- and macroalbuminuria).

## NORMAL RENAL HANDLING OF ALBUMIN

Albumin accounts for ~60% of all plasma proteins; yet only small amounts of albumin are filtered at the glomerulus[14]—although this has recently been questioned.[15] Micropuncture studies suggest a low glomerular filtrate albumin concentration (~22–32 mg l$^{-1}$), consistent with a glomerular filtered albumin load of 4–5 g day$^{-1}$.[16–18] The relative impermeability of the glomerular capillary wall is due to both a size and charge barrier; however, the contribution of the charge barrier has recently been called into question.[19–21]

Filtered albumin is not cleaved in the tubular lumen, but rather binds to a megalin/cubulin receptor complex in clathrin coated pits of proximal tubular cells (predominantly the S1 and S2 segments)[16] (Figure 2a and c), where it undergoes receptor-mediated endocytosis.[22] Inhibitors of 3-hydroxy-3 methylglutaryl-CoA reductase (statins) inhibit this endocytosis, limiting uptake and may result in proteinuria.[23] Endocytic vesicles detach from the apical membrane and deliver the albumin–megalin–cubulin complex to a sorting endosomal compartment, where the albumin dissociates (due to the low pH), and megalin/cubulin are recycled to plasma membrane. Albumin subsequently reaches the lyzosomal compartment, where it is cleaved and the amino acids reabsorbed or peptide fragments 'regurgitated' into the tubular lumen.

## RENAL PATHOLOGY IN DIABETIC KIDNEY DISEASE

To understand the mechanisms underlying the inability of diabetic kidneys to prevent urinary protein leakage, we must first identify the renal histological abnormalities in DKD (Figure 3). Kidneys are typically enlarged as a result of hypertrophy of tubular cells and of all the glomerular cell types (glomerulomegaly). The characteristic histological features on renal biopsy include thickening of the glomerular basement membrane (GBM) and mesangial expansion (Figure 3). GBM thickening, the earliest detectable feature,[24] is due to the deposition of mostly normal GBM constituents

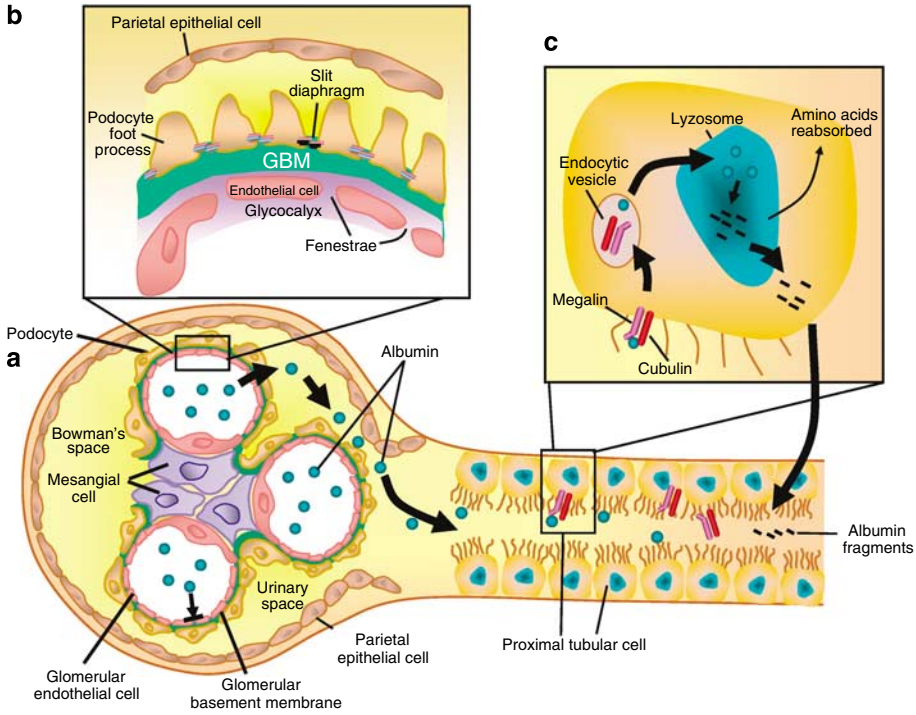


**Figure 2 | Normal renal handling of albumin.** (**a**) Normal glomerulus and proximal tubule. The individual cells and constituents of the glomerulus and proximal tubules are shown. Albumin (represented by green spheres) normally remains within the capillaries of the glomerular tuft, and does not escape into the urinary (Bowman's) space. (**b**) Glomerular filtration barrier. This barrier comprises the innermost glomerular endothelial cells, GBM, and outermost podocytes; and serves to serially limit albumin escaping from the capillary loops. Fenestrae within specialized endothelial cells are covered by a negatively charged glycocalyx. Podocytes attach to the outermost aspect of the GBM by foot processes, between which are proteins comprising the size barrier slit diaphragm. (**c**) Proximal tubule. The albumin that is physiologically filtered at the level of glomerulus into the urinary space is taken up by the megalin/cubulin receptor lining the brush border of proximal tubular cells. Albumin is internalized by vesicles, and upon lysozyme action, the resultant fragments are either reabsorbed or secreted back into the tubular lumen as albumin fragments.

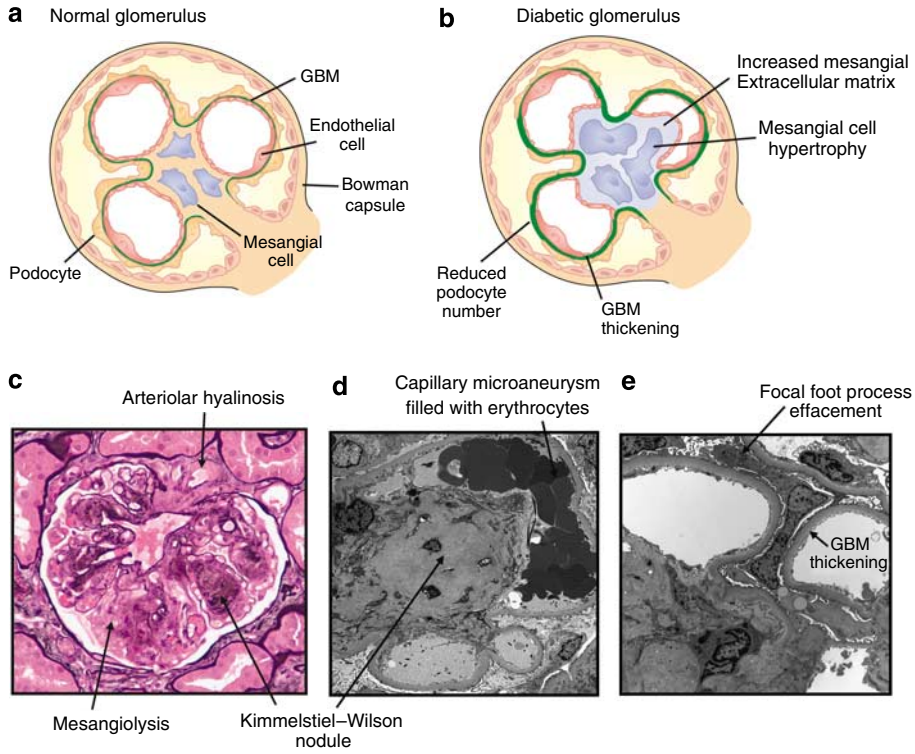


**Figure 3 | Characteristic glomerular changes of DKD.** (**a**) Normal glomerulus. Cells of the glomerular tuft (mesangial cells, endothelial cells, and podocytes) and extracapillary glomerulus (parietal epithelial cells) are shown, along with the GBM. (**b**) Diabetic kidney. In the diabetic kidney, characteristic glomerular changes include thickening of GBM and mesangial expansion (due to increased mesangial matrix and increased mesangial cell size due to hypertrophy). The filtration surface is therefore reduced due to mesangial expansion, leading to reduced glomerular filtration rate. Podocytes undergo several changes, including a reduction in cell number as illustrated. (**c**) Light micrograph showing mesangial changes. Mesangiolysis leads to disruption of the normal mesangial cell architecture. The hallmark of DKD is a localized increase in extracellular matrix proteins resembling a nodule (called Kimmelstiel–Wilson nodule). In addition, arterioles are injured in DKD, with characteristic hyalinosis, leading to a reduction in lumen size. (**d**) Electron micrograph showing mesangial cell changes. A large Kimmelstiel–Wilson nodule is shown encroaching upon the capillary lumen. The capillary architecture is normally supported by mesangial cells. This function is disrupted due to mesangiolysis, resulting in capillary lumen expansion and the formation of microaneurysms. (**e**) Electron microscope of glomerular filtration barrier in DKD. This figure shows that two layers of the filtration barrier are abnormal. The GBM is thickened, and the shape of podocytes is abnormal (effacement). (Pathology images courtesy of Jolanta Kowalewska.)

including type IV collagen α3, α4, and α5 chains, likely produced by podocytes. Mesangial expansion is mostly secondary to increased extracellular matrix proteins (type IV collagen (α1 and α2 chains), type V and VI collagen, laminins, and fibronectin), and to a lesser degree, mesangial cell hypertrophy.[25] Over time, there is disruption and disintegration of the normal glomerular architecture due to mesangiolysis, leading to microaneurysm formation, and the development of the characteristic Kimmelstiel–Wilson nodules is seen (Figure 3c–e). The mesangial changes (increased mesangial fractional volume) lead to a decrease in filtration surface area and have been most closely correlated with a reduced GFR.[25] By contrast, it remains unclear, how mesangial cell injury *per se* causes significant proteinuria (see below). That GBM thickening and mesangial expansion occur in long-term diabetes without albuminuria suggests that these changes may not be the primary mechanisms of proteinuria.[26–29]

Electron microscopy evidence for podocyte injury in DKD includes foot process widening and effacement, and reduced podocyte number. Glomerular endothelial cell injury is not a prominent pathological finding in DKD, although reduced endothelial fenestration has been described.[30] Hyaline deposits between the GBM and endothelial cell (hyaline caps) and along the parietal surface of Bowman's capsule (capsular drops) may also be present. Finally, glomerular changes of DKD are typically associated with prominent vascular lesions (arteriosclerosis and arteriolar hyalinosis) and tubulointerstitial injury (thickening of the tubular basement membrane; tubular cell hypertrophy) with progressive interstitial fibrosis at later stages.

## GLOMERULAR FILTRATION BARRIER: A SERIES OF RESISTORS TO PROTEINURIA

The renal pathologic findings described above do not readily explain proteinuria (urinary albumin excretion $>30\,\mu g\,day^{-1}$) in DKD. Albuminuria/proteinuria results from defects in the glomerular filtration barrier, although abnormalities in tubular albumin reabsorption may also contribute. The glomerular filtration barrier comprises a series of resistors (or layers) separating the blood side (glomerular capillary) from the urinary (Bowman's) space.

These include the innermost fenestrated glomerular endothelium, the middle GBM, and the outermost podocyte (also called the glomerular visceral epithelial cell) (Figure 2b). Each layer likely contributes uniquely to the impermeability to albumin, although recent studies suggest that the podocyte slit diaphragm may be the predominant barrier to albuminuria. Seminal dextran sieving studies by Brenner and others[31–33] suggested a size, charge, and conformational glomerular barrier, although technical concerns regarding the use of dextrans have led to some aspects of this model being questioned.[34] Several theoretical models of macromolecular transport across the glomerular filtration barrier have since been proposed. A bimodal pore-size model seems to most accurately fit the polymer sieving data,[35,36] where numerous restrictive pores exist (diameter 37–48 Å) whereas fewer large pores (60–80 Å) function as a shunt pathway. Charge and size selectivity may be changed independently of each other suggesting different sites or mechanisms. A gel membrane model has also been suggested recently.[18]

### Molecular size and sieving

There is little debate about the role of molecular size on glomerular permeability. Low molecular weight proteins (mw < 40 kDa) are essentially freely filtered,[16] whereas, high molecular weight proteins (mw > 100 kDa) are almost completely restricted.[36,37] Note that the molecular weight of albumin is 69 kDa. In patients with diabetes and low-grade albumin excretion (< 300 mg per 24 h), no change in size selectivity (radius of pores, spread distribution of small pores, or magnitude of large pores) has been reported, suggesting that alterations in the charge barrier may be responsible.[38,39] As the magnitude of albuminuria increases, there is an increase in large pores, and an increase in albumin passage through small selective pores is described that becomes more evident at higher degrees of proteinuria.[38–40]

### A disputed charge barrier

The significance of a glomerular charge barrier remains debated. This was first proposed on the basis of tracer studies showing that anionic ferritin was restricted from entering the GBM, whereas cationic ferritin reached the podocyte slit diaphragm.[41] Further studies showed that compared with negatively charged or neutral dextran, positively charged dextrans were more permeable.[31,32,42] More recent studies using charged and uncharged Ficolls have questioned the existence of a glomerular charge barrier.[19–21] The site of any anionic charge barrier has been considered to lie within the GBM (see below); however, anionic sites have also been demonstrated on podocytes (podocalyxin)[41,43] and the endothelial glycocalyx.[41,44]

### Hemodynamic factors in diabetes may cause proteinuria independent of structural kidney damage

Proteinuria/albuminuria may be increased by Starling type forces across the glomerular capillary due to increased renal plasma flow and/or increased glomerular capillary hydrostatic pressure (predominantly mediated by angiotensin II). Filtration fraction, a measure of glomerular hydraulic pressure, is elevated in patients with albuminuria in type I[45] and II diabetes.[46] Acute changes in blood pressure have been shown to reduce albumin excretion in the short term.[47] By contrast, seminal studies by Anderson, Hostetter, and Brenner showed that chronic elevation in intraglomerular pressure leads to progressive glomerular injury, thereby augmenting proteinuria by further damaging the filtration barrier.[48–51]

Given that we believe the majority of proteinuria in DKD is directly due to one or more abnormalities in the layers of the glomerular filtration barrier (Figure 2b), we will focus on how defects in each of these constituents cause proteinuria.

## THE GLOMERULAR ENDOTHELIUM: FIRST RESISTOR TO PROTEINURIA

### Structure and function of the normal glomerular endothelium

The highly specialized glomerular endothelium lining the glomerular capillaries differs from other endothelial beds. First, they have characteristic fenestrations (60–100 nm in diameter) that serve to facilitate ultrafiltration.[52] Second, they are covered by a cell surface coat called the endothelial glycocalyx[44] comprising a network of proteoglycans with negatively charged glycosaminoglycan side chains forming an extracellular matrix. This links to soluble plasma proteins forming a dynamic layer that is undergoing constant turnover. Thus, from a functional standpoint, the endothelial fenestra may facilitate high hydraulic permeability, counterbalanced by the overlying glycocalyx, which helps to restrict the filtration of macromolecules. The glomerular endothelium has not until recently been considered to have prominent restrictive properties. Ferritin tracer studies have suggested that the fenestrated endothelium is mostly porous to proteins that tend to accumulate under the GBM.[53,54] However, if the glomerular endothelium does not play a prominent role in the filtration barrier, the issue of clogging of the filtration barrier and the question of how large amounts of filtered albumin are returned to the circulation need to be answered.[55]

### Abnormalities in the glomerular endothelium cause proteinuria in diabetes

*(i) Endothelial cell injury.* Systemic endothelial dysfunction is prominent in type I and type II diabetes,[56] manifest by increased endothelial permeability,[57] impaired nitric oxide production,[58] upregulation of adhesion molecules, and the development of a pro-thrombotic phenotype. The Steno hypothesis considers that albuminuria in diabetes may simply reflect the kidney manifestations of systemic endothelial dysfunction.[59] Indeed, albuminuria has been associated with an increased systemic permeability to albumin in both type I[60] and type II diabetes.[61]

The mechanisms underlying systemic endothelial permeability remain to be fully determined, but likely include

hyperglycemia *per se*, glycated hemoglobin, dyslipidemia, inflammatory cytokines, reactive oxygen species, and activation of the renin–angiotensin system (reviewed in Rask-Madsen and King).[62] Despite this, endothelial injury is not a prominent feature of DKD on biopsy.[63]

***(ii) Defects in the glomerular endothelial glycocalyx.*** Recent data suggest a more significant role for the overlying endothelial cell glycocalyx in the development of proteinuria.[64–67] Enzymatic cleavage (hyaluronidase and heparanase) of hyaluronic acid and chondroitin sulfate in mice decreases the thickness of the negatively charged endothelial glycocalyx and is associated with an increased fractional clearance of albumin.[68,69] Notably, the clearance of neutral Ficolls (molecular weight similar to albumin) remained unchanged, suggesting an alteration in the glomerular charge, not size barrier. Preliminary studies in diabetes suggest that the systemic (sublingual capillaries) glycocalyx volume is decreased in longstanding type I diabetes by 50% and is further decreased in type I diabetics with albuminuria,[70] suggesting that hyperglycemia may increase glycocalyx permeability independent of changes in glycocalyx volume.[71] The effects of diabetes on the glomerular glycocalyx remain unknown, although it is tempting to speculate that similar abnormalities may exist.

### VEGF and angiogenesis in the development of proteinuria in diabetic kidney disease

Recent studies have confirmed increased glomerular vascularity and endothelial cell proliferation at early stages of diabetic nephropathy.[72,73] Vascular endothelial growth factor (VEGF), a pro-angiogenic factor, is prominently produced by podocytes.[74] Although VEGF may signal within the podocyte in an autocrine fashion (via VEGFR1),[75,76] VEGF protein primarily appears to cross the GBM and to act on glomerular endothelial cells (via VEGFR1 and VEGFR2) to promote endothelial cell survival and to induce the formation of fenestra, which enhance glomerular endothelial permeability. Studies suggest that the systemic inhibition of VEGF may lead to proteinuria. Anti-VEGF antibody or VEGF receptor antagonists used in cancer trials have been associated with proteinuria.[77] Soluble VEGFR1 (sFlt-1) receptor has also been identified in patients with preeclampsia and proteinuria.[78] Notably, local inhibition of VEGF in the kidney impairs the glomerular filtration barrier. Using recombinant technology with a Cre-loxP system, Quaggin and colleagues[79] have shown that podocyte-specific VEGF-deficient mice fail to form a glomerular barrier (absent glomerular endothelial cells) resulting in embryonic lethality. By contrast, loss of one VEGF-A allele leads to glomerular endothelial cell swelling (endotheliosis), nephrotic syndrome, and renal failure by 9–12 weeks.[79] The mechanisms of proteinuria secondary to VEGF deficiency in this model remain unclear, although the absence of WT1, nephrin, and VEGF suggest that podocyte loss may be significant.[79] It should be recognized that the overall regulation of VEGF appears to be critical, as studies also suggest that increased VEGF activity may be associated with a form of collapsing glomerulopathy and proteinuria.[79]

***Is there a role for VEGF in DKD?.*** In early stages of DKD, VEGF expression is actually upregulated within the glomerulus, specifically within the podocyte, and the VEGF receptor is similarly induced.[80] *In vitro*, podocyte expression of VEGF is increased by high glucose,[81] angiotensin II,[82] and glycated albumin.[83] Could these elevated levels of VEGF be related to proteinuria? Just as VEGF deficiency is biologically important, increased VEGF activity increases endothelial permeability, an effect which may be abrogated by ACE inhibition.[84] Additionally, VEGF may have autocrine effects on podocyte function leading to proteinuria.[79] Blockade of VEGF in experimental models of DKD has been shown to improve both the early functional changes (hyperfiltration and albuminuria)[85] and the later structural changes (GBM thickening and mesangial expansion).[86] By contrast, at later stages of DKD, VEGF expression decreases, which has been shown to correlate with albuminuria,[87] and may be secondary to podocyte loss.[88] On the basis of available published data, the precise role of VEGF in DKD is not clear at this time. The authors believe that while VEGF is likely to play a significant role in proteinuria, further definitive studies are needed in DKD before we can draw definitive conclusions.

## THE GLOMERULAR BASEMENT MEMBRANE: A PARADOX OF THE THICKENED, YET LEAKY SECOND RESISTOR

Because of the characteristic thickening of the GBM in DKD (Figure 3e), early studies focused almost exclusively on this glomerular structure to explain the proteinuria. The normal GBM is a 300–400-nm thick gel-like structure (90% water), which arises during development from the fusion of two distinct basement membranes (one derived from glomerular endothelium and other from the glomerular epithelium). It consists predominantly of type IV collagen, laminin 521 ($\alpha5\beta2\gamma1$), nidogen, and heparan sulfate proteoglycans (HSPGs).

### Heparan sulfate proteoglycan and the GBM charge barrier: challenging conventional wisdom!

***A positive view of HSPG.*** If the GBM does constitute a charge barrier, evidence has suggested that this may be due to loss of heparan sulfates, the sulfated glycosaminoglycan side chains of HSPGs. Studies supporting the hypothesis that GBM heparan sulfate contributes to the charge selectivity of the glomerular capillary wall include (i) administration of a monoclonal anti-heparan sulfate antibody to rats resulted in massive proteinuria[89] (ii) removal of heparan sulfate by enzymatic cleavage (heparanase) resulted in increased GBM permeability,[90] and (iii) a reduction in heparan sulfate immunostaining has been described in various glomerular diseases, which correlate with the degree of proteinuria.[91,92] In DKD, a reduction in immunostaining for heparan sulfate has been described in patients with both type I[93–95] and type II diabetes,[91,92] which inversely correlates with the degree of

proteinuria (reviewed in Raats *et al.*).[96] Studies have also described a reduction in the HSPG core protein agrin.[97,98]

Alterations in HSPG levels lead one to next ask how this matrix constituent of the GBM is regulated? It must be recalled that the GBM is an acellular structure, and, thus, any changes to its structure (that is, matrix proteins) is due to changes in the cells it supports (glomerular endothelial cells and podocytes). The decreased HSPG expression in more advanced disease may be due to either decreased HSPG synthesis by the podocyte or endothelial cell or increased degradation. Decreased heparan sulfate synthesis (by $^{35}$S-sulfate incorporation) has been described in some,[99,100] but not all studies.[101,102] *In vitro*, decreased podocyte synthesis of the core protein agrin may be caused by both a high ambient glucose[103] and angiotensin II.[104] ACE inhibition prevents HSPG loss in diabetic rats and decreases albuminuria.[100] In addition, heparan sulfate in diabetes may be undersulfated with a reduced anionic charge.[99,105] Decreased activity of glucosaminyl *N*-deacetylase/*N*-sulfotransferase, a key enzyme in the sulfation of heparan sulfate, has been described in the glomeruli of STZ rats.[106,107]

Increased enzymatic degradation of heparan sulfate (heparanase) has been described in type II DKD and in STZ rats.[108] High ambient glucose increases the expression of heparanase in podocytes.[109] Heparanase may be inhibited by heparin(oids), which ameliorate proteinuria in several animal models of proteinuria. In patients with DKD, glycosaminoglycan therapies, including sulodexide and enoxaparin, which can inhibit heparanase, have been shown to reduce proteinuria and may provide a new avenue for treatment if clinical outcome studies prove positive.[110,111]

***A negative view of HSPG.*** The majority of the human studies described above were performed in more advanced DKD. More recent studies have shown that the development of albuminuria in early DKD is not associated with changes in GBM heparan sulfate expression or sulfation status.[112] Notably, infusing heparanase III into rats clearly removes HS from the GBM without the development of proteinuria[113] and indeed, may even block the protein passage across the GBM.[114]

It is the view of the authors that the decrease in HSPG is likely both functional and a 'biomarker' of glomerular cell injury. Although decreased HSPG appears to be a common finding in more advanced DKD, it remains unclear if this is a contributing factor to the development of proteinuria or a consequence of podocyte (or endothelial cell) injury.

### A conundrum: how does a thickened GBM promote proteinuria?

As described earlier, thickening of the GBM is a hallmark of DKD, and the degree of thickening has been shown to correlate with proteinuria.[26] The thickening is due to the accumulation of extracellular matrix; however, GBM thickening may begin as early as 2 years following the onset of diabetes, prior to albuminuria, suggesting that this may be a nonspecific result of hyperglycemia.[25,115,116]

Regardless, what contributes to the increased GBM matrix accumulation? The likely culprit is the podocyte. Exposure of podocytes *in vitro* to high glucose or angiotensin II leads to a pro-matrix phenotype, with increased synthesis of new type IV collagen chains.[75,117] Moreover, evidence for reduced degradation also exists in that there is decreased production of matrix-degrading enzymes (matrix metalloproteinases),[118] and/or an increase in their inhibitors (tissue inhibitors of metalloproteinases).[119] In human DKD, a reduction in matrix degradation has been described, partly due to decreased matrix metalloproteinases, an effect ameliorated by ACE inhibition.[120] Together, the increased production and reduced degradation favor matrix accumulation, seen clinically as thickened GBM. The role of the glomerular endothelial cell, which also lines the GBM, is unclear as it is related to the thickening process.

Finally, how could an increase in GBM thickness result in proteinuria? At first consideration, an increase in GBM thickness might be expected to reduce protein transit. However, careful analysis shows that the GBM thickening occurs in an irregular manner with areas of thinned GBM. Possibly more importantly, the GBM structural changes may affect the adjacent cellular elements, reducing cell binding and promoting cellular (in particular, podocyte) detachment.

## PODOCYTES: THE FINAL FRONTIER TO PROTEINURIA

Although the mesangial cell has been considered to be at the epicenter of injury in DKD, there is an increasing and convincing body of research in the past few years documenting that injury to podocytes leads directly to proteinuria/albuminuria in diabetic nephropathy.[9,121] Moreover, injury to podocytes also underlies progressive glomerulosclerosis in diabetic nephropathy, and, thus, a decline in GFR.

### A size and charge barrier

The third component to the glomerular filtration barrier, considered by many to be the most important, is the podocyte (Figure 2b). These are terminally differentiated cells lining the outer aspect of the capillary loops in a very complex architecture of interdigitating foot processes.[122] Podocytes serve several important functions including synthesis and perhaps maintenance of the underlying GBM, counteraction of capillary hydrostatic pressure, and as a critical member of the filtration barrier. The latter probably is the most important function with regard to proteinuria and some consider the podocyte to be the 'last frontier' protecting against protein/albumin loss into the urinary space.

Structurally, podocytes have a cell body, from which primary and secondary foot processes arise. These foot processes are interdigitating and the narrow gaps (30–40 nm) between neighboring processes are bridged by the glomerular slit diaphragm. The slit diaphragm is a highly specialized gap junction with small pores, permeable to water and solutes but relatively impermeable to plasma proteins. The unique and specialized shape of podocytes is supported by an actin cytoskeleton, which not only serves a static function but

which also allows the podocyte to continuously and dynamically alter shape. Podocytes are anchored to the GBM by integrins (predominantly the α3β1 integrin) and α- and β-dystroglycans.

How do podocytes normally prevent proteinuria? Similar to glomerular endothelial cells and GBM, podocytes are also negatively charged on their apical membrane domain owing to surface anionic proteins such as podocalyxin, podoplanin, and podoendin. The negative charge helps limit passage of negatively charged molecules (like albumin). However, podocytes also serve as a size barrier to proteinuria, predominantly due to the properties of the slit diaphragm. The changes in size and charge properties of podocytes occurring in DKD will now be discussed in detail in the context of their contribution to the development and magnitude of proteinuria.

### Decreased podocyte number (podocytopenia)

Podocytes, like most cells, require a critical number to perform their normal functions. Although adjacent podocytes and their foot processes overlap to some extent, they essentially are a monolayer of cells on the urinary side of the GBM. Thus, one can easily envisage that if there is a focal area (or areas) in the glomerulus where podocyte number is reduced from whatever cause (these are discussed below), this would create a specific area of vulnerability when it comes to preventing protein loss (Figure 4). Reduced podocyte number leads to proteinuria by the following mechanisms: (i) an overall loss of negative charge, due to an overall decrease in podocalyxin protein available to serve as a charge barrier; (ii) disruption of the normal architecture of the podocyte monolayer. As such, slit diaphragm integrity and cell–cell connections are altered, and over time, 'gaps' are noted in areas of reduced podocyte number. This leads to the inability of podocytes to function as a size barrier, leading to the unimpeded passage of protein and other molecules into the urine. In addition to proteinuria, the reduced podocyte number may fail to mechanically support the glomerular capillary loop and may lead directly to glomerulosclerosis and loss of renal function.[123]

What is the evidence for reduced podocyte number in diabetics? A decrease in podocyte number has been shown in patients with both type I and type II diabetes mellitus.[124–127] Although podocyte number may be normal at early stages,[127] in some instances, reduced podocyte number precedes the onset of clinically detectable albuminuria and/or proteinuria by several years.[124] A European study suggests that podocyte density, rather than podocyte number, may be more predictive of albuminuria.[128] Experimental and clinical studies have also shown a direct correlation between the extent of the decrease in podocyte number and the magnitude of proteinuria.

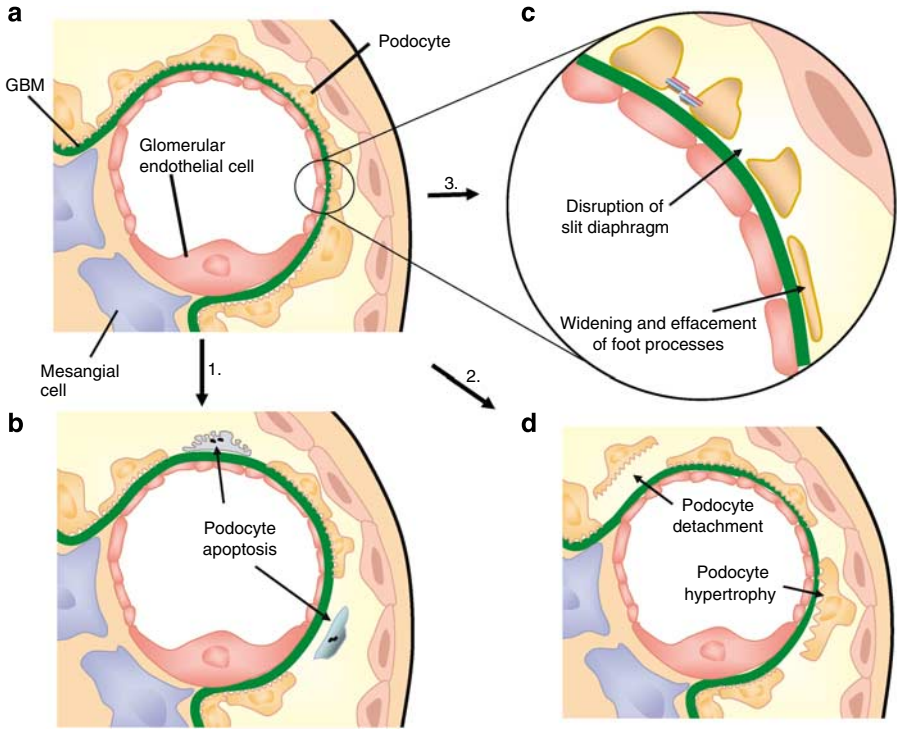


**Figure 4 | Podocyte abnormalities in DKD.** (**a**) Normal podocyte architecture. (**b**) Some podocytes undergo apoptosis, resulting in reduced cell number. Albumin can then leak through areas denuded of podocytes. (**c**) The normal architecture of the slit diaphragm is disrupted (by either reduced levels of specific proteins and/or relocalization of these proteins) leading to a loss of size barrier. Podocytes also undergo changes in shape due to effacement. (**d**) The normal function of podocytes is also limited by individual cells increasing in size (hypertrophy) and detachment leads to reduced overall podocyte number.

### Causes of reduced podocyte number

The causes of reduced podocyte number in diabetic nephropathy are multifactorial, and an active area of study. These will be discussed below.

(i) *Podocyte detachment* (Figure 4d): There is substantial evidence documenting podocyte detachment with their subsequent appearance in the urine described as podocyturia.[129–131] One study described podocyturia in 53% of microalbuminuric and 80% of macroalbuminuric type II diabetics, but was absent in those with normal albumin excretion.[132] Moreover, many of these cells were viable podocytes, suggesting a primary problem with their ability to stay attached to the underlying GBM. Reduced expression of the $\alpha 3\beta 1$ integrin, the predominant integrin tethering the podocyte to the underlying GBM, appears to contribute to podocyte detachment.[133] The authors speculate that the substantial changes in GBM architecture in diabetes might also pose an unfavorable 'footing' to which podocytes attach, thus favoring detachment.

(ii) *Podocyte apoptosis* (Figure 4b): Apoptosis (programmed cell death) of podocytes may be another important cause of podocytopenia in diabetes and has been shown to coincide with the onset of albuminuria.[134] Apoptosis has been difficult to document in all podocyte diseases with the current techniques that are available as the cells slough off into the urine. For cells to undergo apoptosis, the normal balance of pro-survival vs pro-apoptotic signals is tipped. Although several mechanisms might underlie apoptosis in diabetes, oxidative stress has been implicated as an important and even potentially treatable cause.[134]

(iii) *Inability to proliferate and restore podocyte number*: Podocytes are terminally differentiated cells and thus typically do not proliferate *in vivo*. Under normal (unstressed and nondiseased) states, this poses no threat. However, in the setting of loss such as detachment or apoptosis, the relative inability of these cells to proliferate and 'replace' their neighbors leads to an eventual decline in number. Thus, relative lack of appropriate podocyte replication may be another contributing factor to reduced podocyte number in diabetes. Studies have shown that alterations in specific cell-cycle regulatory proteins might prevent podocyte proliferation (reviewed in Shankland).[135] Candidates include an increase in expression and action of the cyclin-dependent kinase inhibitors p21 and p27, which prevent cell-cycle progression.[136] Moreover, there may also be a change in the cell phenotype in diabetes, where rather than proliferating, podocytes undergo hypertrophy. This causes an increase in cell size (not an increase in cell number). How this maladaptive response contributes to proteinuria is not well defined.

### Slit diaphragm abnormalities

As stated earlier, the major size barrier to the passage of proteins and other macromolecules is the slit diaphragm (Figures 2b and 4c). This has been extensively reviewed elsewhere.[137–139] In brief, the 'gaps' between adjacent foot processes are bridged by highly specialized and specific proteins, which form an intricate network such that pores form that are slightly smaller than the size of albumin, thus serving as a size barrier. Moreover, proteins of the slit diaphragm are not only simply structural, but also function to signal to other proteins within the body of the podocyte. For example, nephrin, CD2AP, and podocin also serve a survival function for podocytes. One can, thus, readily appreciate how alterations in the structure and/or function of these proteins lead to not only abnormalities in the size barrier, but also to the overall integrity of podocytes.

A critical component of the glomerular slit diaphragm is nephrin, with an expression pattern restricted to podocytes in the kidney. Any alterations in levels or molecular makeup of nephrin directly lead to podocyte abnormalities by limiting the normal size barrier properties, by augmenting apoptosis, and by altered cytoskeletal functions. Nephrin is shed into the urine of diabetic patients with micro- and macroalbuminuria,[140] and nephrin staining is decreased in both patients with type I and type II diabetes mellitus, correlating with broadening of the foot processes.[141–143] Interestingly, nephrin has recently been shown to be also essential for vesicular docking and insulin responsiveness of podocytes.[144] Insulin responsiveness may be important to enable a physiological response in terms of allowing podocytes to rapidly metabolize glucose to facilitate structural remodeling required to respond to changes in filtration pressure.

### Foot process widening and effacement

Foot process widening is found at early stages of diabetic nephropathy and results in a decrease in the number of slit pores per unit length of GBM.[145] Foot process width has been shown to correlate with the extent of albuminuria,[146] and, importantly, may be prevented by RAS blockade.[147] This raises the question of how might foot process widening contribute to albuminuria? First, changes in the slit diaphragm morphology may simply change the shape of the size barrier, thereby allowing albumin/protein to make their way into the urinary space. The decrease in slit diaphragm length associated with widened foot processes may impede the filtration of water and lower GFR. If at the same time protein permeability remains unchanged, then the amount of protein relative to water would increase in the urinary space and the increased protein concentration may exceed the reabsorptive capacity of the tubular epithelial cells leading to proteinuria.[148]

Effacement or simplification of podocyte foot processes is an active process with the retraction of the foot processes into the cell body resulting in large areas of flattened epithelium covering the capillary loop (Figures 3e and 4c). The exact molecular mechanisms resulting in effacement remain

unclear, although proposed mechanisms include disruption of the podocyte actin cytoskeleton,[149,150] loss of slit diaphragms,[151] or impairment of the podocyte–GBM interaction.[152,153] It is not clear, however, that foot process effacement alone can explain proteinuria, and indeed may be simply a consequence of podocyte injury. The podocyte flattening covers wide expanses of the GBM and the total area for ultrafiltration is probably much decreased, although focal epithelial defects may allow increased protein flux across these denuded areas. It should be noted that there are several experimental models where proteinuria occurs in the absence of foot process effacement,[154–158] and, similarly, effacement can occur in the absence of proteinuria.[159]

#### Cytoskeletal changes in podocytes

(i) *Altered cytoskeleton*: There is recent evidence for cytoskeletal changes of podocytes in diabetes. For example, high glucose and advanced glycosylation end products decrease the expression of α-actinin-4.[160] Since α-actinin plays an important role in glomerular filtration as a key molecule of the podocyte cytoskeleton, reduced expression may contribute to proteinuria. For example, a reduction in α-actinin has been shown to precede podocyte foot process effacement and proteinuria in an experimental model of nephritic syndrome.[150]

(ii) *Reduced negative charge function*: Loss of negative charge on podocyte may be another mechanism in the development of proteinuria in diabetes. Normally, podocytes are covered with a negatively charged glycocalyx consisting of sialoglycoproteins including podocalyxin and podoendin. Recent experimental studies have shown a decrease in podocalyxin expression in podocytes in response to high glucose.[161] The resulting loss in negative charge would make it easier for negatively charged albumin to escape into the urinary space.

In summary, there are a number of podocyte-specific changes that occur in DKD, that likely are very important events in the development and persistence of albuminuria and proteinuria. These include a decrease in podocyte number/density, widening of the foot processes, shortening of the slit diaphragm/loss of slit diaphragm proteins, changes in actin cytoskeleton, and decrease in negative charge. The mediators and factors likely responsible for the abovementioned podocyte changes in diabetes mellitus will be discussed below.

## THE MESANGIAL CELL: A LESSER ROLE IN THE PROTEINURIA

#### Reduced GFR and mesangial cells

Mesangial expansion and the development of the Kimmelstiel–Wilson lesion are perhaps the best known histological characteristics of DKD (Figure 3). Indeed, for many years, it was thought that changes in mesangial cells were central to the clinical findings of DKD. These changes include (i) an increase in mesangial cell size due to hypertrophy (not due to increased cell number), and (ii) an increase in the extracellular components due to an increase in a variety of extracellular matrix proteins. Studies estimate that the cellular component accounts for one-third of the mesangial expansion, and the extracellular component the remaining two-thirds. The mechanisms accounting for these mesangial changes are summarized in several elegant reviews.[8,162,163] The increase in cellular and extracellular mesangial components correlates with, and likely causes, a decrease in GFR by encroaching upon neighboring capillary loops, and with time, reducing the capillary surface area for filtration.[164,165]

Although mesangial expansion clearly leads to a reduced GFR, one is left asking does mesangial expansion also cause proteinuria. It is the view of the authors that mesangial expansion *per se* is not sufficient to cause proteinuria directly based on several lines of evidence. First, mesangial expansion is an early change in DKD and begins prior to the onset of albuminuria.[115] Second, DKD is described both in animal models (Cohen rat)[166] and in humans,[4,28,167] in the absence of proteinuria, but in whom prominent mesangial expansion is noted on biopsy. Notably, the Cohen rat does not seem to pass through an early hyperfiltration phase, and, therefore, glomerular hypertension and accompanying podocyte injury may not develop.

The authors believe that mesangial injury rather leads to secondary changes in the filtration barrier, which then results in proteinuria. First, DKD may be associated with mesangiolysis leading to the development of microaneurysms and endothelial cell injury. Second, we would also speculate that with the anatomic disruption in the capillary loops from microaneurysmal dilatation that there is increased stress–tension generated on podocytes. The analogy would be that of a balloon (capillary loop) being inflated. With increasing distention of the balloon, there is increasing tension on the walls, and a critical pressure leads to disintegration. Third, pioneering work by Brenner and colleagues showed that reduced nephron mass causes increased intraglomerular capillary pressure in the remaining nephrons. We would therefore argue that as glomeruli undergo increasing sclerosis, in large part due to the accumulation of mesangial extracellular matrix proteins in DKD, other less affected glomeruli (or less affected loops of a single glomerulus) experience increased glomerular pressure. Thus, glomerular hypertension will lead to podocyte injury (with proteinuria), and, subsequently, focal glomerulosclerosis and renal dysfunction. Taken together, while mesangial expansion in DKD clearly reduces GFR, it is unlikely to directly cause proteinuria, but may do so indirectly by generating glomerular hyperfiltration.

## TUBULOINTERSTITIAL INJURY: AN OVERLOOKED CONTRIBUTOR TO PROTEINURIA

As discussed earlier, there are often substantial tubulointerstitial changes seen on renal biopsy in diabetics, and it is well established that tubulointerstitial scarring is perhaps the best

predictor of renal survival in both diabetic and nondiabetic disease.[168,169] The reader is referred to excellent reviews on the causes of tubulointerstitial injury in disease, and how this reduces kidney function.[170,171] How might tubulointerstitial injury itself either cause proteinuria, or augment proteinuria that is primarily due to abnormalities in the glomerular filtration barrier? We can appreciate that interruption of this protein reabsorption process in the proximal tubule may lead to proteinuria, although interstitial injury *per se*, without an increase in glomerular protein filtration, would not be expected to cause nephrotic range proteinuria. Of note, an alternative view suggests that, even with normal glomerular function, there are very large amounts of albumin filtered at the glomerulus and that protein retrieval in the proximal tubule is a high volume process and a prime regulator of urine protein excretion.[15] In this model, classic glomerular diseases are associated with proximal tubular injury leading to reduced proximal reabsorption of albumin, and, as a result, the development of nephrotic range proteinuria. We believe that tubulointerstitial injury augments urine protein excretion by impairing the reabsorption of filtered (glomerular) protein. Although not definitive, the bulk of the data would suggest that 4–5 g of albumin are filtered at the normal glomerulus each day.[16–18] In diabetes, proximal tubular reuptake of protein may be impaired by high glucose,[172] TGF-β,[173] or angiotensin II.[174] With the development of glomerular disease, tubulointerstitial injury is enhanced and the ability to reabsorb protein is further reduced. The increased 'glomerular' proteinuria can easily overwhelm the capacity of injured tubular cells to reabsorb the filtered protein load. Taken together, the magnitude of proteinuria is now markedly increased due to this 'compounding' effect of both glomerular and tubulointerstitial injury. Of note, there is a direct correlation between the degree of tubulointerstitial scarring and the extent of albuminuria,[175] and it is likely that each of these variables serves to amplify the other.

## CONCLUSION

Proteinuria (increased urinary albumin excretion) in patients with diabetes is a signature feature of DKD. Although progressive renal impairment due to glomerular and tubulointerstitial scarring has been described in the absence of proteinuria, the two typically occur together. The mechanisms underlying proteinuria, and glomerular and tubulointerstitial fibrosis may overlap in part. Proteinuria in DKD is predominantly due to disturbances in the glomerular filtration barrier, consisting of the glomerular endothelial cell, the GBM, and the podocyte. Although it is tempting to identify the podocyte as perhaps the predominant component of this barrier, it is becoming clear that each layer of resistance within the filtration barrier contributes uniquely to the overall permselectivity preventing proteinuria. Thus, injury to one or more layer in DKD results in the onset of proteinuria, and persistent proteinuria may result from either a lack of adequate repair and/or injury to further components of the filtration barrier. Tubulointerstitial injury may also contribute to proteinuria by impairing proximal tubular protein reabsorption. Proteinuria, along with other factors, may lead to progressive glomerulosclerosis and tubulointerstitial fibrosis, with a subsequent decline in GFR (Figure 1). The studies highlighted in this review provide hope to our patients that extensive efforts are underway aimed at uncovering the mechanisms underlying proteinuria in DKD so that specific therapies can be developed to reduce the burden of kidney and cardiovascular disease.

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