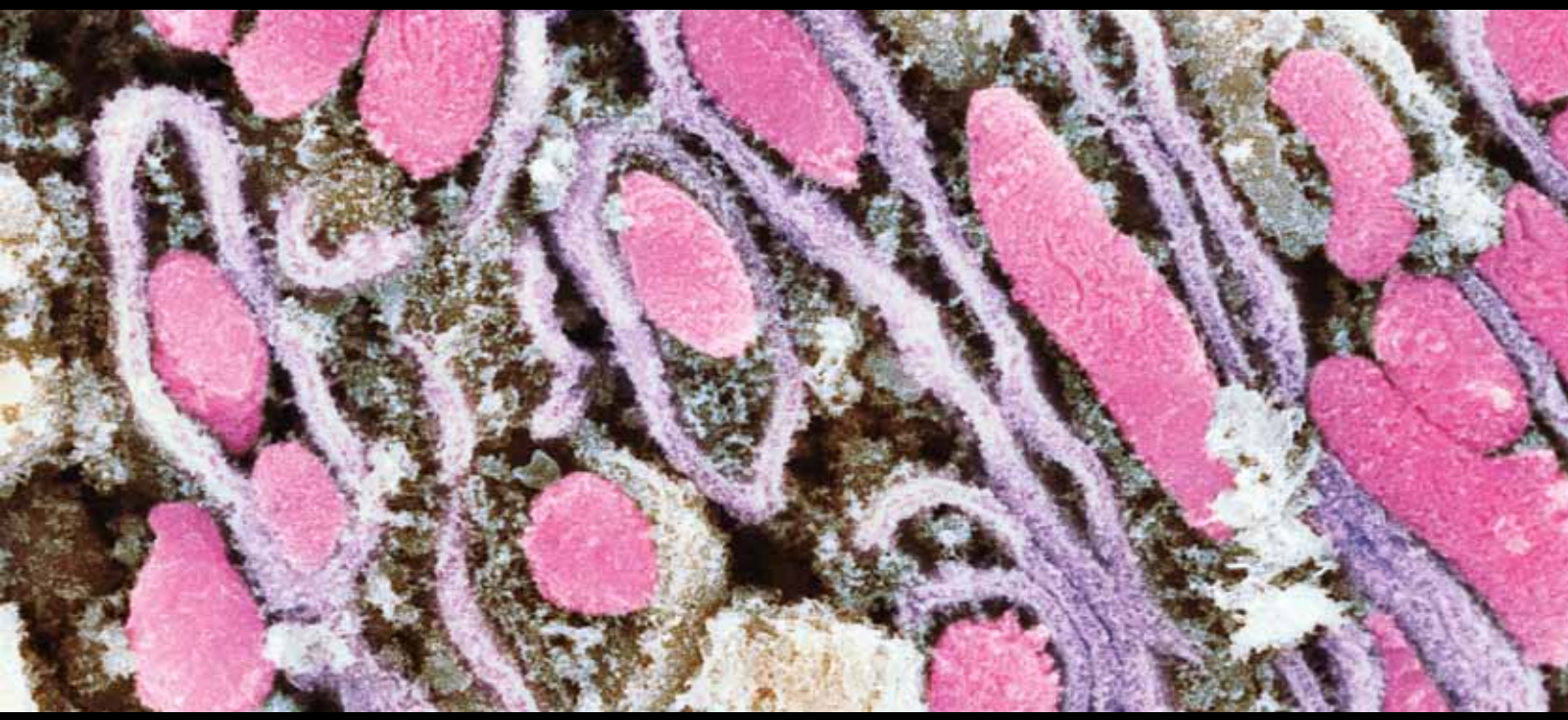
International Journal of Nephrology

Proteinuria: From Molecular to Clinical Applications in Glomerulonephritis

Guest Editors: Claudio Bazzi, Omran Bakoush, and Loreto Gesualdo



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Editorial Board

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Editorial

Proteinuria: From Molecular to Clinical Applications in Glomerulonephritis

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Glomerular diseases including diabetic nephropathy are the leading cause of ESRD worldwide. Proteinuria, the hallmark of renal damage in glomerular diseases, is dependent on two main factors: the alteration of the glomerular filtration barrier and its three layers (glomerular endothelial cells, basement membrane, and visceral epithelial cells (podocytes)), and the impairment of proteins reabsorption by proximal tubular epithelial cells. In recent years there has been a great increase of knowledge of the molecular structure of glomerular filtration barrier and of the molecular mechanisms involved in tubular reabsorption of proteins.

In this special issue the structure of the glomerular filter and the importance of glomerular talk in maintaining the integrity of the glomerular filtration barrier are reviewed by M. Menon et al. A. Tojo and S. Kinugasa reviewed the albumin glomerular permeability in normal and disease status and present their views of a possible mechanism of selective proteinuria in nephrotic syndrome of minimal change disease. A. Zhang and S. Huang summarized several molecular defects responsible for dysfunction of the glomerular filtration barrier. J. E. Toblli et al. overviewed the alterations of glomerular endothelial cells, basement membrane and podocytes, the possible relationship between glomerular proteinuria and tubulointerstitial damage, and described less and more recent approaches to reduce proteinuria. The review of J. R. Machado et al. summarizes the most important molecules involved in the pathogenesis of nephrotic syndrome. Galactose-deficient IgA1 is the hallmark of IgA nephropathy; in a cohort of 40 pediatric patients with biopsy-proven IgAN, a research group from the Le Bonheur Children's Hospital found no association between albuminuria and the galactose-deficiency, a finding that may question the pathologic role of galactose-deficiency in IgAN. The possible therapeutic efficacy of inhibition of mammalian target of rapamycin (mTOR) in primary mesangioproliferative glomerulonephritis was discussed by H. Trimarchi et al. who suggested prospective clinical trials. B. Zhang and W. Shi reviewed the therapeutic effects of cyclosporine A (CsA) in glomerulonephritis and evaluated the data in support of a nonimmunologic antiproteinuric effect of CsA dependent on a direct stabilization of podocyte cytoskeleton. The review by A. Cohen-Bucay and G. Viswanathan discusses nine additional urine biomarkers that may offer better prediction for the course of diabetic kidney disease progression than urinary albumin. The authors call for further longitudinal studies to validate the clinical value of these biomarkers to overcome the limitations of albuminuria.

The great increase in knowledge of molecular biology of glomerular filtration barrier and tubular reabsorption of proteins has not been matched as yet by outcome prediction improvement and therapeutic advances. There is a need for further studies for better understanding the pathogenesis of proteinuria and the clinical value of the different urine biomarkers in diagnosis and management of patients with chronic glomerular diseases.

Finally special thanks to the authors and the reviewers for their efforts to provide to the nephrology community a concise up-to-date knowledge on the pathogenesis and management of proteinuric kidney disease.

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Review Article

The Glomerular Filtration Barrier: Components and Crosstalk

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The glomerular filtration barrier is a highly specialized blood filtration interface that displays a high conductance to small and midsized solutes in plasma but retains relative impermeability to macromolecules. Its integrity is maintained by physicochemical and signalling interplay among its three core constituents—the glomerular endothelial cell, the basement membrane and visceral epithelial cell (podocyte). Understanding the pathomechanisms of inherited and acquired human diseases as well as experimental injury models of this barrier have helped to unravel this interdependence. Key among the consequences of interference with the integrity of the glomerular filtration barrier is the appearance of significant amounts of proteins in the urine. Proteinuria correlates with kidney disease progression and cardiovascular mortality. With specific reference to proteinuria in human and animal disease phenotypes, the following review explores the roles of the endothelial cell, glomerular basement membrane, and the podocyte and attempts to highlight examples of essential crosstalk within this barrier.

1. Proteinuria

Urinary protein excretion in the normal adult humans is less than 150 mg/day. Persistent protein excretion greater than this merits further evaluation. Proteinuria is strongly associated with progression of kidney disease [1]. Furthermore, proteinuria has proven to be an independent risk factor for all-cause and cardiovascular mortality [2]. Proteinuria can be glomerular resulting from an impairment of the glomerular filtration apparatus, tubular from diminished tubular resorption of low-molecular-weight proteins, and overflow—where the resorptive capacity is overwhelmed by large loads of filtered proteins [3, 4]. Although some studies have suggested that the filtration barrier is more permeable to albumin than previously reported [5, 6] thus attributing a more significant role for the proximal tubules in determining the magnitude of proteinuria, more recent studies have disputed those findings and corroborated classical measurements of the glomerular sieving coefficient derived from micropuncture experiments [7, 8]. For the purposes of this paper, we will restrict our discussion to molecular and structural pathomechanisms of glomerular proteinuria.

2. Glomerular Filtration Slit Diaphragm: A Multicomponent Apparatus

The filtration apparatus is complex; its integrity is maintained by an interplay of all participating cell types and constituents [9]. The glomerular filtration barrier (GFB) is freely permeable to water, small- and midsized solutes in plasma, yet maintains considerable size and charge selectivity for proteins and larger molecules. This barrier has three major components: the fenestrated endothelial cell, the glomerular basement membrane (GBM), and the podocyte with their "slit diaphragms". In all pathologic glomerular proteinuria, there is increased filtration of macromolecules (typified by albumin) across this barrier. Injury to this apparatus can be pathogenetically classified as resulting from inherited and acquired causes (Table 1). Inherited human diseases presenting with defects in the GFB have been shown to involve abnormalities of proteins important for the maintenance of podocyte cytoskeleton [10], glomerular basement membrane integrity [11], and glomerular endothelial function [12]. Possibly owing to its specialized structure and abundant blood flow, the glomerulus is uniquely susceptible to both immunological and hemodynamic injury [13]. In addition to

Table 1: Classification of glomerular injury by pathomechanism.

(a) Inherited

Proteins involved in podocyte cytoskeletal integrity [4]
Podocin (AR), nephrin (AR), CD2-associated protein (AD), transient receptor potential cation-6 (AD), α-actinin-4 (AD), phospholipase-Cα1 (AR), tetraspanin CD-151 (AR), and Wilm's tumor-1 (AD)
Basement membrane proteins [9, 11]
Alport's syndrome-α-4 collagen-5 (XL~85%); Alport's syndrome-α-4 collagen-3/4 (AR or AD~15%), thin Basement membrane disease (AD), and laminin-β2 (AR)
Proteins involved in endothelial/microvascular integrity [12, 15]
Complement regulatory proteins—complement factors H and I (AD), membrane cofactor protein (AD), and complement C3 (Gain-of-function)
Lysosomal storage diseases—metabolic injury [9]
Fabry's disease (XL)

(b) Acquired

Immune	Nonimmune	Idiopathic
Immune complex mediated [13, 15]	*Hemodynamic injury* [10]	*Unclear mechanisms*
In situ formation or circulating complexes (postinfectious glomerulonephritis—streptococcal,	Hypertension	Minimal-change disease [22]
hepatits B/hepatitis-C-associated GN, systemic lupus erythematosus,	Adaptive hyperfiltration (nephron loss)	Membranous nephropathy [14]
and IgA nephropathy) idiopathic MP GN (types I and III)	*Other*	Primary FSGS [10]
Antibody-mediated [13]	Metabolic-hyperglycemia in diabetes	
Anti-GBM antibody disease	Deposition disease-Amyloidosis	
T-cell-mediated injury [13]	Toxic—Pamidronate, NSAIDs, D-pencillamine, and gold	
Antineutrophil cytoplasm antibody vasculitis, most glomerulonephritides	Infectious—HIV [10]	
Complement-mediated injury [12, 15]		
MPGN type II, atypical HUS		

Inheritance pattern—autosomal recessive (AR), dominant (AD), and X-linked (XL).

discussing the roles individual components in the GFB play in glomerular function in health and disease, we will attempt to highlight the accumulating evidence for significant interdependence and cross-talk within this unit (Figure 1).

3. Roles of the Podocyte

Podocytes are terminally differentiated, highly specialized epithelial cells of mesenchymal origin located on the urinary aspect of the GFB. The surface of podocytes is covered by anionic glycocalyx, constituted mainly by podocalyxin [14]. Podocytes are characterized by their foot processes, a network of interdigitating cellular extensions (primary, secondary, and tertiary), which support the glomerular capillary loop from the visceral aspect of the Bowman's space and interact at specialized cell-to-cell junctions called slit diaphragms [9]. Slit diaphragms contain proteins that are typically found in tight junctions (i.e., zona occludens-1 [15]) as well as adherens junctions (catenins, P-cadherin [16]). The critical roles of the integral proteins of the slit diaphragm and foot processes have been strongly implied by their association with familial nephrotic syndrome in humans (Table 1) and the renal phenotypes observed in knockout animals. In vitro studies on podocytes have been limited by the inability to reproduce the in vivo podocyte cytoskeletal phenotype in cell culture. Nephrin is a transmembrane protein member of the immunoglobulin superfamily protein found in the slit diaphragm [17]. Its interaction with nephrin (homologous) from an adjacent podocyte or neph-1 (heterologous) is hypothesized to be the "pore" of the slit diaphragm [18]. The intracellular domain of nephrin interacts with podocin and other regulatory proteins to facilitate actin polymerization [18, 19]. The polymerization and specific arrangement of actin filaments is the key to foot

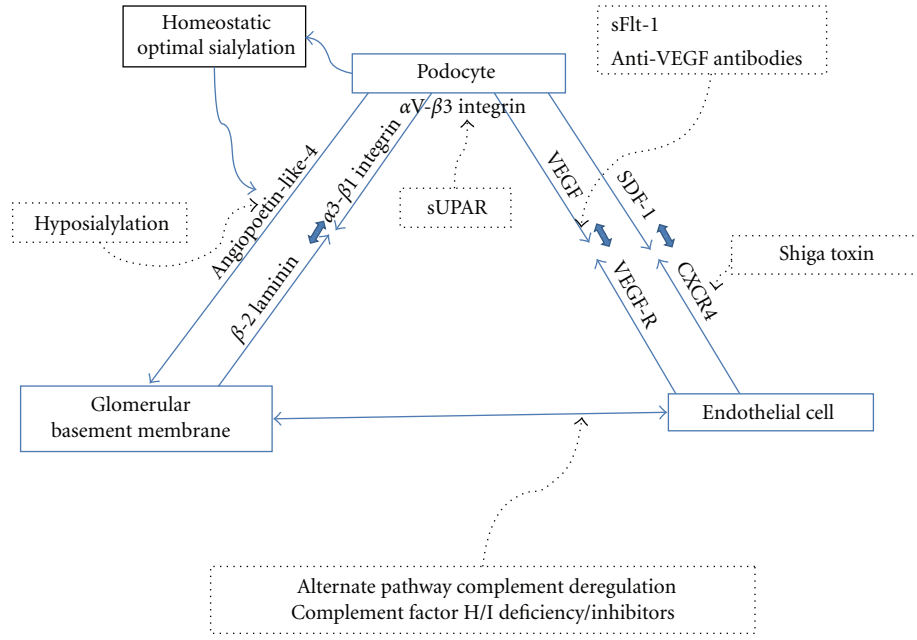

Figure 1: Components of the Glomerular filtration barrier with examples of crosstalk. This figure summarizes important signaling interactions between the 3 key components of the GFB and their putative involvement in human disease models (dotted arrows). Podocyte $\alpha 3$-$\beta 1$ integrin interacts with GBM β-2 laminin; hyposialylated-podocyte-secreted Angp-4 may have an effect on the GBM and the endothelial cell in MCD models. sUPAR, a potential circulating "serum factor," affects podocyte αV-$\beta 3$ integrin in primary FSGS. Podocyte-endothelial cell interactions are affected by VEGF/VEGF-R blocking agents in preecclampsia while SDF-1-CXCR4 pathway is perturbed by Shiga toxins in HUS. Alternate pathway complement dysregulation and consequent endothelial components are central to MPGN and atypical HUS.

process architecture. This is supported by the identification of hereditary nephrotic syndrome with foot process abnormalities resulting from single-gene mutations in a number of actin-binding proteins involved in actin polymerization such as α-actinin-4 and inverted formin 2 [20, 21].

The podocyte slit diaphragm is the final barrier in the GFB to filtration of macromolecules. Distortion of foot process architecture resulting in foot process effacement (FPE) or flattening is observed in the majority of instances where glomerular proteinuria is present with a few notable exceptions. These exceptions [23, 24] argue that foot process effacement is neither necessary nor sufficient to induce proteinuria. The best example is preecclampsia where nephrotic proteinuria is unaccompanied or variably accompanied by effacement [25]. The reported evidence for FPE without proteinuria is less profuse. Glomerular epithelial protein 1 (GLEPP1) is a receptor tyrosine kinase located on the apical surface of podocytes. Homozygous GLEPP1-knockout mice developed abnormally shaped podocytes with FPE but did not develop proteinuria [26]. Additionally, even in diseases where effacement is classically encountered, its quantification by morphometry does not seem to correlate with the degree of proteinuria [27]. FPE as a reversible event, induced by infusion of polycations and reversed by polyanions was classically demonstrated in rats [28]. This concept of FPE as an ongoing process reversibly initiated by podocytes in response to injurious stimuli is reemerging from multiple observations [29, 30]. The best evidence for near complete reversibility of FPE comes from minimal change disease (MCD) nephrotic syndrome in children and adults upon treatment [31]. FPE in FSGS on the other hand seems to represent a more irreversible event [10]. These could imply that FPE occurs as a consequence of injury to podocytes. It may also suggest that FPE in different diseases though morphologically identical may represent different ultrastructural and cytoskeletal entities. Furthermore, FPE appears to require active actin filament reorganization consequent to intracellular signals and may not merely be a passive response [32]. Deficiency of focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase essential in connecting the actin cytoskeleton with the foot process anchor on the GBM, appears to protect podocytes from developing FPE in response to LPS in podocyte-specific FAK-knockout mice [22]. Upregulation in podocytes of cathepsin L, a protein associated with epithelial cell mobility has also been observed to correlate with FPE [33]. This finding may suggest the adoption of a cytoskeletal phenotype by podocytes undergoing FPE that favors motility, often as a response to injury [33].

The interaction between $\alpha 3$-$\beta 1$ integrin and β-2 laminin, and its link to the podocyte actin cytoskeleton is an important part of the podocyte-GBM interaction. Anti-Fx-1A antibody that causes Heymann's nephritis in rats recognizes $\alpha 3$-$\beta 1$ integrin and causes podocyte detachment [34]. Activation of $\alpha 3$-$\beta 1$ integrin results in recruitment of a kinase, integrin-linked kinase (ILK). When ILK is deleted in a podocyte-specific manner, mice developed proteinuria with foot

process effacement and glomerulosclerosis [35]. On the other hand, blocking TGF-β1 or adriamycin-induced activation of ILK using a highly selective small molecule inhibitor preserved podocyte phenotypes and ameliorated albuminuria [36]. Together, these findings suggest that the regulation of α3-β1 integrin/ILK may be important in optimal podocyte and GFB function. Urokinase-type plasminogen activator receptor (uPAR) is a glycosyl-phosphatidylinositol- (GPI-) anchored protein that is a proteinase receptor for urokinase, together forming a part of the machinery needed for cells to breakdown extracellular matrix proteins and migrate. uPAR has also been shown to be involved in nonproteolytic pathways, mainly through its ability to form signaling complexes with other transmembrane proteins including integrins [37, 38]. Among these is αV-β3 integrin in podocytes at the sites of focal adhesions [38, 39]. UPAR knockout mice appeared to be protected from FPE and proteinuria induced by LPS. Induction of uPAR signaling in podocytes lead to FPE and proteinuria via an αV-β3 integrin-dependent mechanism [39]. More recently, circulating soluble uPAR (suPAR) was found to be elevated in 66% of patients with primary FSGS and high levels appeared to confer a strong risk of recurrence of FSGS after transplant. Furthermore, high suPAR levels in mice were shown to induce FPE and proteinuria via an integrin-dependent mechanism [40].

4. Role of the GBM

Studies using metabolic labeling (experimental argyrosis) have demonstrated that GBM synthesis requires contributions from podocytes and endothelial cells, with mesangial cells playing a role in turnover [41]. The structure of the GBM has been well-characterized both morphologically and from a molecular perspective [42]. Two heterotrimeric proteins, type IV collagen (COL4) and laminin, along with sulfated proteoglycans are the preeminent constituents of the GBM. In homeostasis, β2-laminin principally constitutes the GBM whereas tubular basement membranes have β1-laminin chains [43]. Similarly, GBM COL4 is enriched in α-3, 4, and 5 chains whereas most other basal laminae constitute of α-1 and 2 chains. In the embryonic kidney α-1, 2 chains are still encountered in the GBM but are later completely replaced. Deficiency of COL4 α-5 gives rise to Alports syndrome with the most severe X-linked variant showing extensive GBM lamellations, fragmentations, and progressive glomerulosclerosis [11]. Proteinuria, though encountered is not a prominent pathogenic feature of Alport's syndrome. Autosomally inherited COL4 α-3 and α-4 chain mutations may give rise to recurrent hematuria and show only thin basement membranes on biopsy—akin to thin basement membrane disease. In mice, mutations in α-3 chains leads to absence of α-3/4/5 chains likely because heterotrimer formation with α-4/α-5 chains is disrupted. These mice develop GBM splitting, thinning, and proteinuria with podocyte FPE [44]. β2-laminin in the GBM interacts with and binds to α3-β1 integrin of the basal podocyte membrane as discussed above and in turn, these integrin heterodimers are linked to the podocyte actin cytoskeleton [33, 34]. β2-Laminin deficiency in humans leads to familial nephrotic syndrome with ocular abnormalities—Pierson syndrome [45]. Interestingly, mutant mice with β2-Laminin deficiency upregulate β1-Laminin giving the appearance of a structurally normal GBM but develop proteinuria with foot process fusion [46]. The high specificity of this interaction highlights the role of the GBM in maintaining GFB integrity and podocyte ultrastructure.

Studies using variedly cationic ferritin first demonstrated the anionic nature of the GBM [47]. The anionic charge is mostly conferred by a lattice-like network formed by the sulfated glycosaminoglycan moieties of constituent proteoglycans [48]. Based on the increased permeability of the GFB to ferritin after heparanase treatment, the GBM was initially thought to be the source of charge selectivity in the GFB [49]. This hypothesis has been called into question more recently. Spherical Ficoll/Ficoll sulfate particles of differing negative charge did not show any charge-dependent variation in filtration fraction in isolated GBM preparations [50]. The authors preferred Ficoll as its shape and structure are less alterable with changes in charge, which contrasted with previous studies which had used radio-labelled peptides (albumin) or dextran [51, 52]. Since podocytes are the site of synthesis of heparan sulfate (HS), Chen and colleagues used a Cre-loxP strategy to target the podocyte enzyme, HS polymerase (EXT1) [53]. Mice in whom Cre recombinase expression driven by a podocyte-specific, podocin promoter were crossbred with animals in whom exon 1 of EXT1 was floxed. The resultant mice had dramatically reduced (though not absent) HS staining in their GBM. However, in spite of a demonstrable loss of GBM charge by polyethyleneimine staining, they developed only mild albuminuria and glomerulomegaly at 8 months. There was also no statistically significant difference in body weights compared to controls [53]. Though this model does not take into account endothelial or mesangial synthesis of HS, it does suggest that GBM charge may not be central to GFB permselectivity.

An illustration of the podocyte-GBM interdependence in the GFB has emerged from studies in NS models. Angiopoetin-like-4 (Angp4) is a podocyte-secreted glycoprotein whose transcript levels were observed to be upregulated in nephrotoxic-serum nephritis models. Clement et al. proceeded to generate transgenic podocin-Angp4 rats and mice with podocyte-specific upregulation of Angp4. These mice developed mild proteinuria with effacement of foot-processes. Homozygous rats, however, showed significant and selective albuminuria (100–500-fold, 90% albumin) with diffuse foot-process effacement analogous to human MCD. In puromycin nephropathy models of glomerular injury in rats, Angp4 was upregulated in podocytes. Further, glucocorticoids appeared to downregulate Angp4 and improve clinical parameters. Most interestingly in both mice and rats, Angp4 overproduction was associated with demonstrable loss of GBM charge. In human MCD patients, overexpression of Angp4 was detectable by immunohistochemistry. Hyposialylation of Angp4 residues was prominent and the disease showed improvement after feeding affected animals with a precursor of sialic acid to normalize sialylation. A parallel transgenic rat with adipocyte-specific Angp4

overproduction had no renal phenotype, excluding an effect from circulating Angp4 levels. Based on these findings, the authors hypothesize that podocyte-specific overproduction (with consequent hyposialylation) of Angp4 in response to inflammatory stimuli could progressively accumulate and interfere with GBM-to-podocyte signals causing foot-process effacement and proteinuria. The accumulation of Angp4 in the GBM may also reach the endothelial cells and affect signals to podocytes from them. Since mice which developed relatively mild disease had a similar degree of loss of GBM charge as rats, loss of anionic charge is unlikely to be the primary mechanism in this MCD model [54].

5. Glomerular Endothelial Cell

Due to the presence of overt fenestrations (50–100 nm size; ~20% of surface area), the glomerular endothelium was discounted early as the site of permselectivity in the GFB. Newer studies have refocused attention on the endothelium and its coating glycocalyx layer composed principally of proteoglycans [55–58]. Intralipid injection studies show this glycocalyx layer to extend to around 200 nm into the capillary lumen. Seminal work involving specialized perfusion and fixation techniques demonstrated the glycocalyx "plugs" that appear to cover these fenestrae [56]. Disruption of this glycocalyx layer by hyaluronidase and adriamycin has been shown to induce proteinuria [57]. Recently, arterial hypertonic saline infusion in rat kidneys with resultant displacement of the noncovalently bound particles of this layer was shown to increase filtration of albumin 12-fold [58].

Examples of primary endothelial injury inducing damage to the GFB have been better elucidated within only the last decade. Endothelial cell activation is recognized as a component of many immune-mediated glomerular diseases [55]. From human disease phenotypes, the glomerular endothelium has appeared to be particularly susceptible to complement-mediated injury. In homeostasis, in spite of a low-grade autologous activation of both alternate and classical pathways of complement, it is protected by both soluble and membrane-bound regulators of complement [59]. Acquired deficiency or inactivating mutations of these soluble regulators, notably factor H (CFH), have been associated with a spectrum of glomerular disease, from Dense-deposit disease at one end to atypical hemolytic-uremic syndrome at the other [12]. CFH is a 150 KD plasma protein containing 20 homologous repeats (Complement-control protein domains). While the N-terminal of CFH appears to house the C3b binding site, the C-terminal is responsible for endothelial interaction. N-terminal mutations in CFH and antibodies targeting this moiety appear to induce a Dense-deposit disease phenotype while C-terminal mutations and antibodies cause the development of HUS phenotype [12, 59–62]. Moreover, membrane cofactor protein (MCP; CD46) is a widely expressed transmembrane complement regulator, whose deficiency on the surface of endothelial cells also causes HUS [63]. This again brings forth the carefully regulated role of the endothelium essential to maintaining the GFB in homeostasis.

The essential cooperation within the components of the GFB has been highlighted by the role of vascular endothelial growth factor (VEGF). VEGF-A is secreted by podocytes and localizes to the cell membrane and foot processes [64, 65]. In cultured primary and immortalized human podocytes, Foster et al. observed autocrine effects of VEGF on calcium homeostasis, cell survival, and differentiation [64]. However, podocyte-specific, VEGF-receptor-2 (VEGFR-2-) knockout mice have normal glomerular development and function whereas postnatal whole body VEGFR-2 deletion leads to marked glomerular endothelial cell and microvasculature abnormalities with proteinuria [66]. Thus, greater significance appears to be attached to the paracrine effects it exerts on endothelial cells, diffusing against the direction of filtration. VEGF-knockout mice die during early embryogenesis itself as a result of a failure of vascular formation, even prior to nephrogenesis [65]. Podocyte-specific knockout of VEGF caused hydrops and renal failure with glomeruli showing markedly diminished endothelial cell migration. Additionally, mesangial cells were absent suggesting glomerular VEGF-A is required for mesangial cell migration. Glomerular injury, in the form of endothelial swelling (endotheliosis) and proteinuria, was evident in heterozygotes implying VEGF-A has a dose-dependent role [67]. Furthermore, podocytes in these mice showed evidence of dedifferentiation. In adult glomeruli, VEGF-A also appears to be essential for the maintenance of the fenestrae [65, 67].

The significance of this VEGF-mediated interaction in glomerular disease has been demonstrable in the glomerular lesion accompanying preecclampsia, a syndrome of proteinuria, hypertension and adverse fetomaternal outcomes [25]. Soluble fms-like tyrosine Kinase, a secreted form of VEGF-receptor (sflt-1 or VEGF-R1) competitively inhibits the binding of VEGF to the membrane-bound form of the receptor. It is overexpressed in pre-ecclamptic placental tissue and increased in the serum of these patients. Serum sflt-1 levels decline after delivery [25]. Typically, the glomeruli in affected patients show endotheliosis ("bloodless" glomeruli), subendothelial fibrin deposition with relative preservation of podocyte foot processes. Two elegant experimental models have shown the crucial role of podocyte-secreted VEGF-antagonism in the genesis of these glomerular lesions. Maynard et al. injected pregnant rats (approximating second trimester in human gestation) with adenovirus encoding sFlt-1 to mimic systemic sFlt-1 overproduction. These rats developed glomerular endotheliosis and exhibited hypertension with proteinuria by day 17 (early third trimester) [68]. Eremina et al. developed a tetracycline-inducible, podocyte-specific VEGF-knockout mouse using the Cre-loxP system. Upon stimulation with tetracycline these mice developed proteinuria with the characteristic lesions in glomeruli [69]. Bevacizumab is a humanized neutralizing monoclonal antibody against all human VEGF-A isoforms [70]. A systematic analysis of 7 trials showed that proteinuria developed in 20–60% and hypertension in 3–36% of Bevacizumab-treated patients [71]. Other VEGF-A antagonists have also been implicated [72]. Among reported patients, those with available histology are few and suggest variable preservation of podocyte foot processes. This could

suggest a continuum of injury that begins in the endothelial cells of these patients with the loss or reduction of podocyte VEGF-A. The podocyte dedifferentiation observed in VEGF-null mice and the FPE encountered in some human biopsies may then imply the lack of a VEGF-dependent endothelial signal necessary for proper podocyte function.

Another example of a podocyte-secreted signal acting on endothelial cells is stromal cell-derived factor 1 (SDF-1 or CXCL12). Glomerular endothelial cells express CXCR4, the cognate receptor of SDF-1. Absence of either CXCR4 or SDF-1 gives rise to identical and lethal phenotypes in mice. In the developing nephron, SDF-1 producing podocytes and CXCR4-expressing endothelial cells appear to be aligned adjacent to each other. Further, glomeruli from endothelium-specific, CXCR4-knockout mice showed substantial endothelial cell detachment suggesting a key role for SDF-1/CXCR4 signalling in normal glomerulogenesis [73]. More recently, significant upregulation of CXCR4 transcripts was observed in cultured human microvascular endothelial cells in response to Shigella dysenteriae toxin-1 (STX), the causative agent in diarrhea-associated HUS. Children with documented *E. coli* O157:H7 infection who went on to develop HUS later had 4-fold higher SDF-1 levels than infected individuals who did not develop HUS. Furthermore, mice treated with SDF-1 inhibitor along with STX exposure, showed improved survival and abrogated HUS phenotype when compared to control mice [74]. This implies that the SDF-1/CXCR4 pathway is important in the development of diarrhea-associated HUS.

6. Mesangial Cell: Podocyte Crosstalk in Glomerular Disease

Mesangial cells are specialized pericytes whose primary functions are to provide structural support, regulate blood flow of the glomerular capillaries by their contractile activity, and control the turnover of mesangial matrix. Mesangial cells have been shown to synthesize transforming growth factor-β (TGF-β)—a key mediator in kidney disease progression. They express receptors for and respond to vasoactive substances including angiotensin-II, and many cytokines including TGF-β [75]. Experimentally, the central role of mesangial cells in glomerular development was implied by platelet-derived growth factor (PDGF) deficient or PDGF-β receptor deficient mice which lack mesangial cells and fail to develop glomerular tufts [76, 77]. Rats treated with rabbit antithymocyte serum sequentially develop severe complement-mediated mesangial cell death (with little involvement of other intraglomerular cell types) followed by mesangial proliferation, marked matrix expansion, and renal failure [78]. Further, proliferation of mesangial cells and expansion of mesangial matrix are hallmarks of many glomerular diseases [79].

Mesangial matrix expansion is typical in diabetic glomerulosclerosis suggesting a role for mesangial cells. In in vitro models of glycemic injury to mesangial cells, high-glucose media appear to increase synthesis of collagen I and IV. This effect appeared to be controlled in an autocrine fashion by mesangial TGF-β synthesis and was blocked by anti-TGF antibody [80]. Further, transfer of bone marrow (BM) cells from diabetic db/db mice into irradiated naïve B6 mice induced mesangial matrix expansion and albuminuria. Importantly these mice did not themselves develop diabetes or impaired glucose tolerance. The authors concluded that BM-derived mesangial cell progenitors were responsible for transmitting the diabetic nephropathy phenotype to naïve mice suggesting that mesangial cells exert a key influence in this disease [81].

The cross-talk between mesangial cells and podocytes culminating in proteinuria and progressive renal failure is best exemplified in IgA nephropathy. Polymeric hypogalctosylated IgA molecules appear to be central to IgA nephropathy [82]. Polymeric IgA molecules from patients with IgA nephropathy have been shown to induce phenotypic, secretory and proliferative changes in mesangial cells [83]. TGF-β, tumor necrosis factor-α (TNF-α) and renin-angiotensin-aldosterone axis genes are upregulated in mesangial cells upon polymeric IgA treatment [83]. These IgA-molecules, however, are unable to directly induce podocyte changes in culture. Instead when medium from mesangial cells cultured in the presence of polymeric IgA is added to podocyte culture, it caused decreased expression of podocyte differentiation markers. Furthermore, similarly cultured podocytes showed markedly increased expression of TNF-α and TNF-α receptors 1 and 2 [84]. TNF-α, TGF-β, and angiotensin-II have been shown to be cytokines of central importance in the progression of interstitial fibrosis in IgA nephropathy [83, 85]. Together, these examples of mesangio-podocyte interaction could help devise a therapeutic strategy in these diseases centered around the mesangial cell. In summary, we have attempted to highlight the interdependence among the principal components of the glomerular filtration apparatus that is vital to its integrity. Injury to these individual components or disruption of inter-component relationships seems to bring out both specific and common disease phenotypes often characterized by glomerular proteinuria. Better characterization of the key molecules involved in human diseases along with gene-targeting studies in experimental animals has considerably furthered our understanding of this crucial ongoing intra-glomerular crosstalk and may ultimately make possible specific targeting of these pathways to mitigate and/or treat glomerular disease.

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Review Article

An Overview of Molecular Mechanism of Nephrotic Syndrome

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Podocytopathies (minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS)) together with membranous nephropathy are the main causes of nephrotic syndrome. Some changes on the expression of nephrin, podocin, TGF-β, and slit diaphragm components as well as transcription factors and transmembrane proteins have been demonstrated in podocytopathies. Considering the pathogenesis of proteinuria, some elucidations have been directed towards the involvement of epithelial-mesenchymal transition. Moreover, the usefulness of some markers such as TGF-β1, nephrin, synaptopodin, dystroglycans, and malondialdehyde have been determined in the differentiation between MCD and FSGS. Experimental models and human samples indicated an essential role of autoantibodies in membranous glomerulonephritis, kidney damage, and proteinuria events. Megalin and phospholipase-A2-receptor have been described as antigens responsible for the formation of the subepithelial immune complexes and renal disease occurrence. In addition, the complement system seems to play a key role in basal membrane damage and in the development of proteinuria in membranous nephropathy. This paper focuses on the common molecular changes involved in the development of nephrotic proteinuria.

1. Introduction

A variety of primary and systemic kidney diseases, trigger an excessive protein loss in the urine [1]. Diseases that progress to nephrotic syndrome are grouped into three categories: antibody-mediated diseases (e.g., lupus, membranoproliferative glomerulonephritis, and membranous nephropathy); metabolic disorders (e.g., diabetes, amyloidosis, and Fabry disease) and podocytopathies [2, 3]. The main primary glomerulopathies that course with proteinuria are podocytopathies (minimal change disease and focal segmental glomerulosclerosis), and membranous nephropathy. The podocytopathies are characterized by changes in podocytes. These changes may be at the structural or molecular levels, and some proteins have been shown as a pivot of renal injury and proteinuria development [4].

2. Podocytopathies

Podocyte dysfunction may have idiopathic, genetic, or reactive etiologies. The latter involves response to various insults, including mechanical stress, medications, toxins, viral infections, and as yet unidentified circulating proteins [5]. focal adhesion kinase (FAK) plays an important role in the foot process effacement commonly seen in podocytopathies [6, 7].

Focal segmental glomerulosclerosis (FSGS) involves changes in the slit membrane [8, 9] as well as in the cytoskeleton and cell stress [8–10]. Furthermore, FSGS may occur due to a collapse of the capillary loop, which could be HIV-1 associated [11]. Mutations in *WT1* gene can also cause Denys-Drash syndrome [12]. Alterations in podocyte proteins or mutations in their coding gene play an important

role in the pathogenesis of podocytopathies. Changes in nephrin, as well as in the nephrin homologue Neph1 [13], in CD2-associated protein (CD2AP) [14, 15], in mFAt1 [16], or in podocin [17] have been described. Autosomal recessive changes in *NPHS2* gene causes steroid-resistant nephrotic syndrome [12, 18, 19]. *NPHS2* gene polymorphisms cause proteinuria in patients with minimal change disease (MCD) [20]. It has been proposed that apoptosis, necrosis, or loss of cellular adhesive interaction induce podocyte detachment from the GBM, playing a central role in the FSGS-mediated hyperfiltration process. Modifications in the foot process cytoskeleton may lead to nephrotic syndrome development, being podocalyxin believed to be responsible for foot process stability [21, 22]. Another component of the luminal membrane is a transmembrane tyrosine-phosphatase named GLEPP-1, which might function as a receptor [23] or regulate both the pressure and the filtration rates [24]. The expression of GLEPP-1 seems to be downregulated in patients with FSGS and collapsing glomerulopathy but at normal levels in MCD cases [25]. It has been recently demonstrated that podocalyxin is increased in nephrotic syndromes [26].

The pathogenesis of glomerular sclerosis seen in FSGS might to be caused by an increase in glomerular profibrotic cytokines, such as IL-13 and IL-4 [27–30], whereas other studies suggested the increase of TGF-β levels in this process [30, 31].

The TGF-β pathway controls cellular responses to many chronic glomerular injuries, thereby leading to an increase in the production of extracellular matrix, an increase in podocyte number and area, and apoptosis [32–37]. The TGF-β signaling pathway may act as a key mediator in cellular mechanisms responsible for glomerulosclerosis and interstitial fibrosis [35]. A mediator of TGF-β signaling, Smad7, is strongly expressed in clinical cases of podocyte injury. In vitro culture of mice podocytes in the presence of TGF-β showed that both Smad7 and TGF-SS1 are related to cell apoptosis, suggesting that Smad7 participates in the progressive reduction of podocytes [38].

Some studies have shown that higher renal expression of TGF-β1 would be observed in children with FSGS in comparison to patients with MCD. This finding suggests that TGF-β1 gene transcription in the kidneys may suggest the development of FSGS renal lesions [39].

Evidences suggested that podocytes may succumb the epithelial-mesenchymal transition (EMT) after antigenic encounter. In this phenomenon, podocytes lack their specific epithelial cell markers such as nephrin, P-cadherin, and zonula occludens-1 and acquire markers specific for mesenchymal cells such as desmin, fibroblast-specific protein-1, matrix metalloproteinase-9, type I collagen, α-smooth muscle actin, and fibronectin. These changes may lead to a damage in glomerular filtration barrier, which results in proteinuria [40–42].

Elevated TGF-β1 production might induce the expression of integrin-linked kinase (ILK), a protein that is related to the pathogenesis of many nephropathies that course with proteinuria. The upregulation of ILK in the podocytes may determine the occurrence of EMT in these cells via snail transcription factor induction [43].

Podocytes of patients with FSGS and membranous nephropathy present lower expression of nephrin mRNA than cells from patients with MCD [44]. Apparently, EMT events are more frequently associated to FSGS and membranous nephropathy than to MCD.

Nephrin is an important component of the slit diaphragm. It also functions as a potent recruiter of other proteins to podocyte membrane such as podocin and CD2AP [45]. Intracellular domains of the nephrin protein are tyrosine phosphorylated by Src family kinases [46]. The phosphorylated tyrosine residues of nephrin might bind to Nck adaptor proteins and consequently induce a local polymerization of actin [47, 48]. In adult mice, it has been shown that inhibition of Nck expression in podocytes, promoted a fast induction of proteinuria, glomerulosclerosis, and the morphological changes observed in foot processes. These results suggest that Nck proteins might contribute to keep intact the glomerular filtration barrier in adults [49].

The foot processes are composed by actin cytoskeleton which main components are actin itself, α-actinin, and synaptopodin [50, 51]. Some nephrotic syndromes may present a cytoskeleton reorganization after upregulation of α-actinin [21]. Dominant mutations in α-actinin-4 (ACTN4) gene are associated with FSGS occurrence [52]. The expression of synaptopodin is generally preserved in nephrotic syndromes as MCD, but reduced in FSGS [53]. The expression of other podocyte cytoskeleton-bound proteins, such as the dystroglycans, are kept unaltered in FSGS, but decreased in active MCD [54]. Some studies suggest an important interaction between actin cytoskeleton structure and some components of the slit diaphragm as podocin, nephrin and CD2AP [55, 56].

The nephrotic syndromes are known by their changes in podocytes as the effacement of podocyte foot processes, as well as structural changes in cytoskeleton and molecular reorganization of slit diaphragm [57]. The B7-1 molecule (CD80) is a transmembrane protein commonly encountered only in the cell surface of B lymphocytes and antigen presenting cells [58–60]. Reiser et al. have shown that under stress conditions B7-1 might be expressed on podocytes, which may cause reorganization of actin cytoskeleton and modulation of molecules component of the slit diaphragm [61]. It suggests that B7-1 might be directly involved in the pathogenesis of the nephrotic syndrome.

Sometimes the differentiation between FSGS and MCD cases is very difficult, mainly when renal biopsies present inadequate numbers of glomeruli. In such cases, the typical FSGS focal sclerosis is unable to be evidenced [4]. In these cases, the discovery of other markers is urgently needed. Malondialdehyde is a lipid peroxidation marker induced by oxidative stress that may occur in acute or chronic nephropathies [62, 63]. It has been observed that urinary and serum levels of malondialdehyde as well as its glomerular expression were elevated in patients with FSGS when compared to MCD cases [64, 65]. This indicates that tissue expression of oxidative stress markers should be considered as differential diagnostic tools.

3. Membranous Nephropathy

Membranous nephropathy (MN) is one of the most common causes of the nephrotic syndromes in adults, corresponding to 20 percent of the cases [66–68].

The main pathogenic mechanism involved in MN is the deposition of immune complexes, in subepithelial regions that leads to a progressive thinning of the glomerular capillary [68, 69]. For more than 50 years [66, 67, 69–71], the Heymann nephritis model, was induced in rats immunized with a crude kidney-cortex preparation. The data collected from this model suggested that subepithelial glomerular depositions occur due to the circulating immune complexes, caused by membrane fractions from rat renal brush border [66–69, 71–74]. Additionally, with the advent of the passive Heymann nephritis model, it was observed that rats treated with antibodies directed against brush-border proteins also had the same subepithelial depositions, suggesting that circulating immune complexes are not necessary for this event [75, 76].

Afterwards, megalin was found to be the rat antigen involved in this process. Megalin is expressed in the basal surface of the podocyte foot process, the same subepithelial space where immune complexes are formed. That was the first evidence that podocytes could be engaged with the formation of immune complexes. After the development of genome sequencing techniques, specific megalin epitopes were discovered and were associated with the formation of immune complexes [66, 69, 71–74]. Concomitant with megalin discovery, a new experimental model for MN was established by rabbit immunization with cationic fetal bovine serum. This procedure led to the observation that exclusively immunized animals were capable of having subepithelial IgG and C3 deposits, while the animals that received anionic or neutral serum had complex deposits in the mesangium. This experimental model of Heymann nephritis has been reproduced in dogs, cats, rabbits, rats, and mice [77]. Likewise, proteinuria was more intense in animals that received the cationic serum, showing that podocytes might not play a key role in the formation of immune complexes [72–74, 78]. Renal cortex analysis of mice that developed MN after immunization with fetal calf serum by cDNA microarrays, showed that 175 genes had altered expression in relation to normal kidneys, and metallothionein-1—Mt-1, cathepsin D—CtsD, and laminin receptor LAMR-1-1, previously associated with injury, inflammation, and cell-matrix interactions, were overexpressed. This increase in expression was confirmed by Western blotting, and CtsD and Mt-1 were expressed predominantly in tubulointerstitial compartment and LAMR-1 in glomeruli, distribution evidenced by immunohistochemistry [74].

Other enzymes such as DPP IV, NEP, or aminopeptidase A were also recognized as target antigens for circulating antibodies in animal models. NEP enzyme is located in the Bowman's capsule and proximal tubule in both human and rabbit kidneys. The DPP IV enzyme, however, is also found in podocytes, indicating that these two enzymatic antigens may participate in the pathogenesis of membranous nephropathy [67, 68].

The first human antigen to be linked with an autoimmune cause of the disease was the phospholipase A2 receptor (PLA2R). This antigen is expressed in podocytes and it is a member of the mannose receptor family acting as a transmembrane receptor for secreted phospholipases. The interaction between PLA2R and its specific antibody might potentiate the activation of the complement cascade, which, in turn, might damage the filtration membrane and induce proteinuria [71, 78]. Recent studies have shown an association between the presence of phospholipase A2 receptor (PLA2R) in 70% of patients with MN [79].

The role played by the complement system seems to be essential for the development of the disease. That would be reasonable given the fact that IgG immunoglobulin binding to complement fraction C1q induces proteinuria [66, 69, 71]. Analysis of the glomerular capillary area in kidney biopsies collected from membranous nephropathies demonstrates the presence of C5b-9 membrane attack complexes of the complement system. Animal models demonstrated that podocyte lesions might be mediated by reactive oxygen species (ROS) produced in response to the glomerular membrane damage and the deposition of the immune complexes. These ROS might have their damaging effect on the matrix proteins enhanced by lipid peroxidation. The C5b-9 complex might damage the podocyte DNA directly or through the induction of ROS production. Podocyte lesion might increase the expression of matrix metalloproteinase-9 (MMP-9) in these cells, which might induce collagen IV degradation and alterations in nephrin expression. Thus, lipid peroxidation, complement system activation, and ROS production would provide future therapeutic targets for membranous nephropathy [66, 68, 69, 71, 72]. Another molecule of the complement system which has recently been related to the membranous nephropathy is C4d [80]. C4d is generated by the classical or lectin complement pathway. This fragment is highly stable and covalently binds to cell surfaces. Patients with MN show deposition of C4d in situ and it is believed that this molecule is involved in the pathogenesis of this disease. However, interestingly, such as this deposit is not seen in cases of minimal lesion disease, this molecule has great potential as a tool in the differential diagnosis between these two entities [80].

Immunoglobulin subclasses IgG1 and IgG4 are regularly found in MN, being identically deposited in the glomeruli, but presenting a conflicting expression in patients with antenatal form of the disease. Evaluation of the production profile of IgG1, IgG4, and anti-NEP antibodies have shown that IgG4 alone is not enough to produce nephropathy. Children born from mothers who produced decreased anti-NEP antibody levels, but sustained IgG4 subclass, did not present any renal alteration. On the other hand, children whose mother produced all classes of antibodies did present renal failure at birth. One suitable explanation would be that the Fc portion of the IgG classes would differentially interact with complement system and induce variable cellular lesions [66–68, 70–72, 78].

4. Conclusion

The mechanisms of proteinuria in primary glomerulopathies are complex and depend on all the components of the glomerular filtration barrier. In primary membranous glomerulopathy, some molecules such as megalin and phospholipase A2 receptor have been considered as being the antigens responsible for subepithelial immune complexes, which change the glomerular permeability. In both FSGS and MCD podocytopathies, the molecular changes observed in proteins from the cytoskeleton, cell transmembrane, and slit diaphragm induce foot process effacement and changes in negative charges, resulting in strong proteinuria. The understanding of the mechanisms involved in each clinical entity is extremely important for the best treatment choice and adequate patient followup.

Authors' Contributions

J. R. Machado and L. P. Rocha have contributed equally to this work.

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Review Article

Understanding the Mechanisms of Proteinuria: Therapeutic Implications

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A large body of evidence indicates that proteinuria is a strong predictor of morbidity, a cause of inflammation, oxidative stress and progression of chronic kidney disease, and development of cardiovascular disease. The processes that lead to proteinuria are complex and involve factors such as glomerular hemodynamic, tubular absorption, and diffusion gradients. Alterations in various different molecular pathways and interactions may lead to the identical clinical end points of proteinuria and chronic kidney disease. Glomerular diseases include a wide range of immune and nonimmune insults that may target and thus damage some components of the glomerular filtration barrier. In many of these conditions, the renal visceral epithelial cell (podocyte) responds to injury along defined pathways, which may explain the resultant clinical and histological changes. The recent discovery of the molecular components of the slit diaphragm, specialized structure of podocyte-podocyte interaction, has been a major breakthrough in understanding the crucial role of the epithelial layer of the glomerular barrier and the pathogenesis of proteinuria. This paper provides an overview and update on the structure and function of the glomerular filtration barrier and the pathogenesis of proteinuria, highlighting the role of the podocyte in this setting. In addition, current antiproteinuric therapeutic approaches are briefly commented.

1. Introduction

Proteinuria is considered a major healthcare problem that affects several hundred million people worldwide. In addition, proteinuria is a sensitive marker for progressive renal dysfunction and it is considered an independent risk factor for cardiovascular (CV) morbidity and mortality [1].

Furthermore, it is widely accepted that microalbuminuria (albumin urinary excretion of 30 mg–300 mg/day) is the earliest clue about the renal involvement of diabetes, obesity, and the metabolic syndrome. Interestingly, while microalbuminuria is more predictive of reaching CV end points than kidney end points, macroalbuminuria (total protein urinary excretion >500 mg/day) has been demonstrated to be more associated with reaching kidney end points [2].

However, microalbuminuria can often progress to overt proteinuria leading 10–50% of the patients to end-stage kidney disease development, ultimately requiring dialysis or transplantation. Of similar importance is the observation that even levels of albumin under the microalbuminuria threshold (so-called "high normal") are associated with an increased risk for CV outcomes [3]. Therefore, a reduction or prevention of protein urine excretion is highly desirable.

It is worth remembering that the current staging system for chronic kidney disease (CKD) is based primarily on estimated glomerular filtration rate (eGFR) with lower eGFR associated with a higher risk of adverse outcomes. Moreover, the risks of mortality, myocardial infarction, and progression to chronic renal failure associated with a given level of eGFR are independently increased in patients with

higher levels of proteinuria. In fact, patients with heavy proteinuria but without overtly abnormal eGFR appear to have worse clinical outcomes than those with moderately reduced eGFR but without proteinuria [4]. Although proteinuria is also associated with poor renal outcomes, the current guidelines have been criticized because they do not incorporate information about the presence and severity of proteinuria, an important marker of CKD that is associated with adverse outcomes [5–8].

As the measurement and sampling procedures for proteinuria assessment have not been standardized yet, it is of clinical importance to take into account different types of urinary proteins, albumins, laboratory techniques, and urine sampling methods in order to have the best approach for an individual patient.

Total urinary protein can be assessed using dipstick, precipitation, and electrophoresis methods. Urinary albumin, the predominant urinary protein in most proteinuric renal diseases, can be evaluated using an albumin-specific dipstick, immunochemical techniques, and size-exclusion high-performance liquid chromatography. In addition, urine albumin may be immune-reactive, immune-unreactive, fragmented, and biochemically modified, and assorted laboratory techniques have variable abilities to detect different types of albumin.

Urine specimen for proteinuria assessment can be obtained either from a timed collection or a spot urine sample. Nevertheless, currently spot urine protein- or albumin-to-creatinine ratios are preferred to a 24-hour urine sample in routine practice. Moreover, urinary ratios are also helpful in monitoring changes in the degree of proteinuria in CKD patients [4]. Whereas the assessment of albuminuria in patients with diabetic nephropathy is of paramount importance, proteinuria and albuminuria tests both have a role in nondiabetic kidney disease and in general population screening [9].

It is widely accepted that proteinuric nephropathies seem to progress independently of their initial aggression type leading to irreversible parenchyma damage and end-stage renal disease if otherwise unattended.

The molecular mechanisms that lead to proteinuria and podocyte effacement have been poorly understood for a long time; consequently, targeted therapies have been lacking. Fortunately, an interesting body of data has emerged in this field in the last few years [10]. The discovery of podocyte gene defects that underlie some hereditary proteinuric syndromes has changed our understanding of the relative contributions of the components of the glomerular filter. Additionally, the pathogenic pathways activated in podocytes during proteinuria have been identified. Based on this scenario, therapeutic strategies for controlling urinary protein excretion, which may contribute to delaying or stopping GFR loss, acquire clinical relevance.

2. Physiopathology of Proteinuria: Mechanism and Progression of Renal Disease

The microscopy architecture of the "glomerular filtration barrier" is constituted by three different layers, the glomerular endothelial cell, the glomerular basement membrane, and the visceral epithelial cell or podocyte. Although all of them are important to preserve normal glomerular function, the podocyte, the most differentiated cell type in the glomerulus, seems to be the essential part of the filtration unit (Figure 1).

2.1. Glomerular Endothelial Cell (GEC). One of the characteristics of GEC is the presence of numerous fenestrations. These are round or ovoid transcellular holes through the most attenuated part of the GEC cytoplasm. Since these openings are substantially large in relation to the side of the albumin molecule, a considerable amount of albumin might pass through them. Nevertheless, GEC has a cell-surface layer, namely, "glycocalyx," which in normal conditions impedes the leakage of free albumin as well as other proteins. This glycocalyx is principally composed of proteoglycans and sialoproteins [11]. Biophysical models indicate that fenestral glycocalyx contributes to 50% of the overall hydraulic resistance of the glomerular filtration barrier [12]. Therefore, changes in the amount or composition of glycocalyx within the fenestrae would also have significant effects on GFR. In agreement with this concept, some experimental studies have reported that defects in GEC glycocalyx were associated with proteinuria, which highlights the importance of this complex structure [13, 14].

Several lines of evidence showed that the vascular endothelial growth factor (VEGF), the best characterized angiogenic/vasculogenic factor, is a critical "cross-talk" protein among the components of the glomerular filtration barrier [15]. VEGF, mainly synthesized by podocytes, is required for normal GEC function as clinical and experimental data have demonstrated [16–19]. However, contradictory data emerge from animal models or human kidney biopsies of various glomerulopathies. In minimal change nephropathy (MCN), VEGF, and its receptors were, as well as in diabetic nephropathy, upregulated and correlated with the severity of proteinuria [20, 21]. In patients with MCN and nephrotic syndrome, the urinary VEGF levels are increased and positively correlated with the degree of proteinuria [22].

In addition, renal biopsies from patients with membranous glomerulonephritis (MGN), membranoproliferative glomerulonephritis (MPGN), endocapillary nephritis, and crescentic nephritis presented markedly increased VEGF protein in podocytes [23].

Therefore, the role and/or interaction of podocyte-derived VEGF in glomerular health and disease still remain as open debate.

2.2. Glomerular Basement Membrane (GBM). For many years, the GBM has been considered to have a key role in the glomerular filtration of macromolecules. The essential component in the GBM is type IV collagen, which is early secreted by GEC during fetal development ($\alpha1\alpha2\alpha1$), and is later replaced by the more robust collagen network constituted primarily of $\alpha3\alpha4\alpha5$ heterotrimers secreted by the podocyte [24]. The GBM also contains laminin, nidogen,

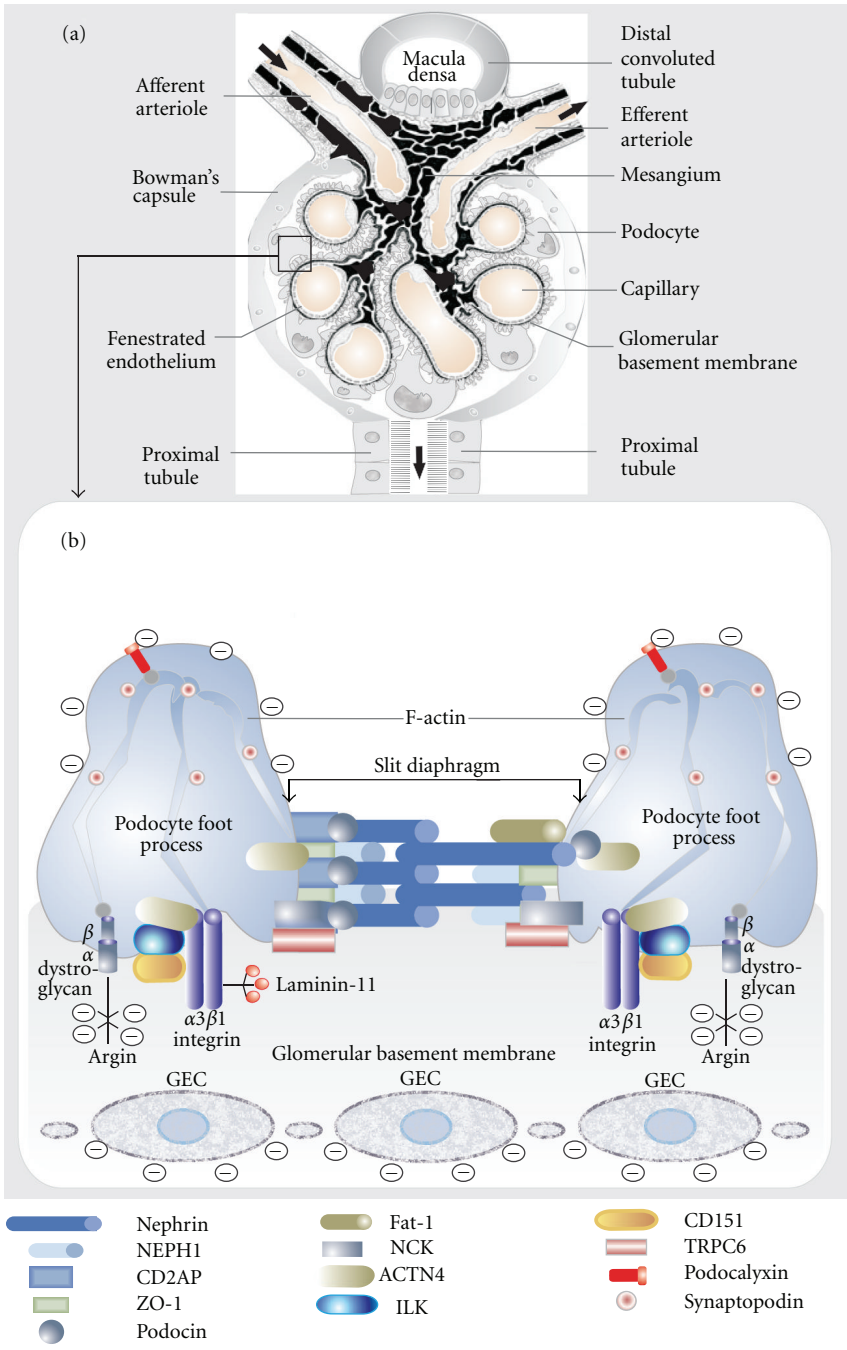
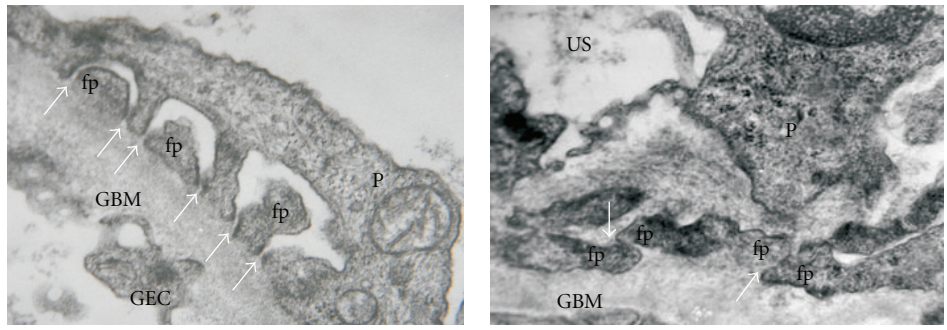


Figure 1: Schematic representation of glomerular filtration barrier components. (a) Illustration of all participant structures in the normal glomerular filtration process. The rectangle indicates the tight relationship between the glomerular capillary wall, glomerular basement membrane, and glomerular visceral epithelial cell (podocyte) which is detailed in the lower panel. (b) Molecular structures of the podocyte and slit diaphragm. Abbreviations: ACTN4: α-actinin-4; CD2AP: CD2-associated protein; GEC: glomerular endothelial cell; ILK: integrin-linked kinase; ZO-1: tight junction protein ZO-1; CD151: tetraspanin CD151; TRPC6: transient receptor potential cation channel 6; NCK: protein adaptor NCK.

and sulfated proteoglycan, components found in all basement membranes, but for some of these classes, the specific isoforms present in the GBM are very different from those found in other basement membranes. Most of these proteins are produced by podocytes and in less proportion by GECs [25].

Type IV collagen is organized as a crosslinked network of triple-helical molecules that primarily provide structural support to the glomerular capillary wall and make little contribution to the size-selectivity or charge-selectivity of the glomerular filter. Emphasizing this concept, mutations in the genes that encode GBM type IV collagens result in collagen



(a) (b)

Figure 2: Glomerular filtration barrier in normal and pathological conditions. (a) A cross-section of a normal human glomerular capillary. Arrows indicate the foot processes with interconnecting ultrathin slit diaphragms. (b) Significant changes in the glomerular filtration barrier in a patient with heavy proteinuria due to a primary glomerulopathy. Note the foot process effacement with alteration in slit diaphragm (arrows), which results in a disarrangement of the functional filter structure. Abbreviations: fp: foot process; GBM: glomerular basement membrane; P: podocyte; GEC: glomerular endotelial cell; US: urinary space. (Transmission electron microscopy. Uranyl acetate-lead citrate original magnification $\times 40,000$.)

α5 (IV) alteration, this leading to the Alport syndrome, where hematuria in conjunction with moderate proteinuria is present [26].

The proteoglycans are heterogeneous molecules composed of a core protein with covalently attached glycosaminoglycan side chains (GAGs). The two best studied proteoglycans in the GBM are perlecan and agrin. They have heparan sulfate as a GAG side chain that provides for at least part of the GBM charge selectivity against the passage of negatively charged molecules into urine. Interestingly, although intravenous administration of heparanases results in increased glomerular permeability to ferritin, which suggests a competent role of heparan sulfate proteoglycans in the filtration barrier, other experimental studies have minimized the actual protein loss [27, 28].

Laminins are heterotrimeric proteins that self-assemble into a network in basement membranes. Laminin-11 (α5, β2, and γ1 chains) is found in mature GBM and connects to collagen IV through nidogen and entactin. The autosomal recessive mutations in laminin β2 gene result in the Pierson syndrome, which is characterized as a congenital nephrotic syndrome accompanied by ocular and neurological defects. Most of the patients with the Pierson syndrome who are diagnosed with nephrotic-range proteinuria rapidly develop ESRD and die in the perinatal period [29].

2.3. Visceral Epithelial Cell (Podocyte). Podocytes are the largest cells in the glomerulus; they have long cytoplasm processes which extend from the main cell body and divide into individual foot processes (pedicels) that attach the cell to the GBM (Figure 2). A considerable number of microtubules and microfilaments are present in the cytosol of podocytes, together with actin filaments, which are particularly abundant in the foot processes (Figure 1). In normal conditions, the distance between adjacent foot processes near the GBM varies from 25 nm to 60 nm. This gap is bridged by a thin membrane coined as "slit diaphragm" (Figure 1).

Podocytes are anchored to components of the underlying GBM via transmembrane cell receptors such as $\alpha\beta$-dystroglycan and integrins, the $\alpha\beta$ heterodimeric proteins that are generally responsible for connecting epithelial cells to basement membranes. $\alpha 3\beta 1$ integrin is the most abundant isoform present in podocytes and is localized exclusively to the basal membrane, linked to the actin cytoskeleton (Figure 1). Furthermore, the α3 chain is necessary for the development of the glomerular capillary tuft. Mice in which the integrin α3 gene is inactivated during podocyte development show massive proteinuria within the first week of life and electron microscopy shows complete foot process effacement and widespread lamination with protrusions of the GBM [30]. The mechanism which explains the presence of proteinuria in integrin-deficient mice is not totally clear. Nevertheless, since $\alpha 3\beta 1$ integrin is a major receptor for laminin, the disruption of the integrin-laminin complex could result in the weakening of the podocyte-GBM interaction and progressive detachment of podocytes, which is associated with proteinuria.

Integrin-linked kinase (ILK) seems to have an essential role in the glomerular filtration barrier. Podocyte ILK was found to be upregulated in human proteinuric glomerular diseases. Additionally, ectopic expression of ILK in podocytes decreased levels of the epithelial markers nephrin and ZO-1, induced mesenchymal markers such as desmin, fibronectin, matrix metalloproteinase-9, and alpha-smooth muscle actin, promoted cell migration, and increased the paracellular albumin flux across podocyte monolayers [31–33]. Another participant in the podocyte-GBM interface is tetraspanin CD151, which has a strong lateral interaction with integrin $\alpha 3\beta 1$. This interaction is important for the adhesion to the GBM as CD151-knockout mice develop proteinuria within a few weeks of birth and electron microscopic examination reveals the presence of lamination and spikes in the GBM as well as focal foot process effacement, though with less severity than that seen in integrin α3 knockout mice [30]. It is worth mentioning that a contractile structure, composed of actin, myosin, α-actinin-4, vinculin and talin, is present

in the podocyte foot processes. This group of proteins is connected to the GBM at focal contacts by the $\alpha 3\beta 1$ integrin complex [34].

Podocytes are also covered by glycocalyx, principally podocalyxin, a sialomucin closely related to CD34 and endoglycan. Through interactions with several intracellular proteins and at least one extracellular ligand, podocalyxin regulates both adhesion and cell morphology. In the developing kidney, podocalyxin plays an essential role in the formation and maintenance of podocyte foot processes, and its absence results in perinatal lethality [35]. In puromycin aminonucleoside nephrosis and protamine sulfate perfusion studies, podocalyxin's negative charge is neutralized, foot process architecture is disrupted, and slit diaphragms are displaced or completely replaced by leaky, discontinuous junctions [36].

The slit diaphragm is one of the major impediments to protein permeability across the glomerular filtration barrier. Consequently, alterations in the cytoskeletal architecture and/or expression of slit diaphragm proteins can be present in most nephrotic disorders. In recent years, the discovery of the molecular basis of the regulation and function of the slit diaphragm structure and its relation with genetic forms of nephrotic syndrome has placed the podocyte as the target cell linked to the development of proteinuria. Nonetheless, from the clinical point of view, it is relevant to distinguish the proteinuria in the case of genetic mutations of slit diaphragm proteins from that which develops in the scenario of arterial hypertension, diabetes, and progressive CKD. In these particular conditions, a GEC alteration with loss of charge selectivity of glycocalyx is probably the first event. This exposes the podocytes to the deleterious effects of albumin and other macromolecules. It is worth emphasizing that in metabolic diseases such as diabetes, albumin may undergo glycation and nitration, which cause substantial structural and functional modifications in its protein configuration [37]. Consequently, the continuous exposition to modified albumin may directly lead to alterations in podocyte function and the disarrangement of slit diaphragm structure.

Both, CD2AP (CD2-associated protein) and NCK (Figure 1) are linker proteins which connect the slit diaphragm to the actin cytoskeleton of podocytes. CD2AP directly interacts with actin (F-actin) and synaptopodin, an actin-bundling protein. Furthermore, CD2AP also interacts with nephrin and podocin in the slit diaphragm (Figure 1). Mutations in CD2AP may cause focal segmental glomerulosclerosis (FSGS) depending on their severity [38]. The interaction with CD2AP might predominate in a steady-state situation, whereas the interaction with NCK proteins could be important during injury and development.

Using diverse techniques as fractionation, immunofluorescence, and immunoelectron microscopy, tight junction proteins such as junction adhesion molecule A, occluding, and zone occludens-1 (ZO-1) have also been demonstrated to be at the slit diaphragm [39].

The slit diaphragm contains transmembrane proteins such as nephrin and Neph1, which are unique to podocytes, as well as proteins such as Fat-1, P-cadherins, and catenins, which are typical of an adherence junction.

Nephrin belongs to the immunoglobulin superfamily whose members are involved in cell-cell adhesion. Nephrin has an important role in maintaining the structure of the podocyte slit membrane, as shown by nephrin-deficient mice which develop proteinuria and foot process effacement [40]. Moreover, the injection of anti-nephrin antibody in animals also results in foot process effacement, and nephrin mutations were observed in patients with the congenital nephrotic syndrome of the Finnish type [41, 42].

It is thought that nephrin binds across the junction to itself or a similar protein called NEPH1 [43, 44]. This cross-junctional binding has been postulated to form a physical sieve that creates a size-selective pore in the slit diaphragm [45] (Figure 1).

Podocin (NPHS2, OMIM 604766), a member of the stomatin protein family, is exclusively expressed in the podocytes and localizes at the insertion of the slit diaphragm (Figure 1). This protein, like nephrin, associates with lipid rafts and recruits nephrin and CD2AP in these rafts ensuring a stable and proper functioning filtration barrier. Podocin dysfunction leads to alterations of the slit diaphragm assembly and to proteinuria in experimental models. NPHS2$^{-/-}$ mice develop proteinuria and massive mesangial sclerosis with enlarged and focally vacuolized podocytes along with a rapid progression to sclerosis with aging [46]. In humans, podocin mutations are mainly associated with the autosomal recessive steroid-resistant nephrotic syndrome [47].

The transient receptor potential cation (TRPC) is a member of a family of proteins involved in the regulation of Ca^{2+} influx. These ion channels can be activated subsequently to either depletion of Ca^{2+} from internal stores or through receptor-mediated processes [48]. TRPC6 (OMIM 603652) is localized to the podocyte cell body, primary processes and in close vicinity to the slit membrane where it interacts with nephrin and podocin (not CD2AP). TRPC6 is also abundantly expressed in mesangial cells [49]. High glucose downregulates the TRPC6 protein, which might contribute to the impaired Ca^{2+} signaling of mesangial cells. This effect may explain the alteration in the mesangial contractile function due to a reduced Ca^{2+} influx observed in diabetic nephropathy [50].

TRPC6 was also found mutated in families with an autosomal dominant form of FSGS [51]. These mutations may cause a gain of function, and consequently an enhanced influx of Ca^{2+}, especially after the activation of the G-protein-coupled receptor AT1 by angiotensin II (Ang II), this resulting in an altered channel regulation or an altered interaction with other slit diaphragm proteins like nephrin and podocin, which leads to proteinuria. In addition to the effects of gain-of-function mutations in the TRPC6 gene, also elevated levels of wild-type TRPC6 protein in some acquired glomerular diseases, like membranous nephropathy and puromycin aminonucleoside-induced albuminuria, may lead to podocyte dysfunction [52]. Recently it has been demonstrated that Ang II participates in the podocyte injury by increasing TRPC6 expression via an NFAT-mediated positive feedback signaling pathway. This finding highlights the crucial role of TRPC6 in the pathogenesis of podocyte injury and proteinuria [53].

Although, nephrin, podocin, VEGF, and synaptopodin are well-known podocyte markers, other proteins expressed in glomerular podocytes, such as glomerular epithelial protein 1 (GLEPP-1) and podoplanin, have also been involved in a number of glomerular diseases. Reduction in GLEPP-1 has been found associated to podocytopathies not only in biopsies of patients with IgA nephropathy and FSGS [54] but also in women with preeclampsia [55]. In addition, GLEPP-1 is considered a contributor to podocyte's foot process structure regulation. GLEPP-1 is a 132 kDa membrane protein tyrosine phosphatase with a large extracellular domain containing eight fibronectin-type-III-like repeats, a hydrophobic transmembrane segment, and a single protein-tyrosin phosphatase domain. Podoplanin, a 43 kDa integral membrane glycoprotein localized on the surface of rat podocytes, is downregulated in puromycin nephrosis [56]. Additionally, in the spontaneously proteinuric Dahl SS rat, segmental loss of podoplanin expression accompanied proteinuria and preceded widespread podocyte alterations for several weeks [57].

The Mammalian Target of Rapamycin (mTOR) and Autophagy in Podocytes. The mTOR is an evolutionarily conserved protein kinase [58]. The mTOR forms two distinct functional multiprotein kinase complexes, termed TOR complex 1 (TORC1) and TORC2 [59], which mutually phosphorylate different substrates and regulate a wide array of essential cellular processes including translation, transcription, and autophagy. mTOR is active in several types of cancer and plays a role in a variety of other serious human diseases, including diabetes, neurodegenerative disorders, and polycystic kidney disease.

Recent studies suggested that mTORC1 inhibition by rapamycin or everolimus can favourably modify glomerular diseases, such as minimal change disease [60], focal segmental glomerulosclerosis [61], membranous nephropathy [62, 63], crescentic glomerulonephritis [64], and diabetic nephropathy [65]. In diabetic animals rapamycin could prevent GBM thickening, glomerular hypertrophy, mesangial expansion, and renal macrophage [65]. Although the inhibition of mTORC1 activity attenuates pathological phenotypes of various different renal diseases in rodent models, rapamycin treatment has been associated with proteinuria in humans [66, 67]. Recent studies suggest that disruption of the autophagic pathway may play a role in the pathogenesis of proteinuria in patients treated with mTOR inhibitors [68].

Autophagy is an important homeostatic and quality control mechanism that maintains cellular integrity and has been shown to be essential for long-lived postmitotic cells, such as podocytes [69]. Autophagy refers to the process of self-degradation of cellular components in which proteins and organelles are sequestered and modified within cytosolic double-membrane vesicles, the autophagosomes, and subsequently delivered to the lysosome [70].

Although the mechanism by which mTOR regulates autophagy remains unclear, the induction of autophagy by mTORC1 inhibition is largely responsible for the potent effect of starvation on cell size, and rapamycin induces autophagy in a wide variety of cell types and species by inhibiting the activity of mTORC1 [71].

Whereas physiological level of mTOR activity inhibits autophagy in podocytes, mTOR reactivation allows autophagolysosomal reformation and the cycle of autophagy to complete itself. On the other hand, mTOR inhibition disrupts the autophagic pathway at two points. First, it relieves chronic suppression, resulting in activation and enhanced autophagy. Furthermore, mTOR inhibition will also lead to suppression of the reformation of lysosomes and autophagosomes, ultimately resulting in an accumulation of autolysosomal vesicle damaged intracellular organelles such as mitochondria and cell death [68].

Since autophagy has been recently identified as a crucial factor for glomerular maintenance and glomerular aging [69], it is very reasonable to assume that autophagy could be an interesting and novel therapeutic target for the treatment of glomerulopathies. However, in any attempt at manipulating podocyte autophagy therapeutically, it will be important to take into account the dynamic nature of the changes that occur in the autophagic system and related protein degradative pathways during the course of glomerular disease.

APOL1 and Podocyte. Recently, using the strategy of mapping by admixture linkage disequilibrium (MALD), two groups identified MYH9 as one of the genes underlying the ethnicity-driven health disparity in both end-stage renal disease (ESRD) [72] and FSGS [73].

Variation in MYH9 was estimated to account for about 70% of ESRD in non-diabetic African American patients [74]. Upon further examination, however, it was suggested that the true association with ESRD was with the apolipoprotein L-1 gene (APOL1), due to both the stronger statistical association with that gene and the lack of identification of causal functional variants in MYH9 [75–77].

Localization of APOL1 within the podocyte was demonstrated using immunofluorescent confocal microscopy to colocalize APOL1 with markers for podocytes (GLEPP1 and synaptopodin) in normal human kidney sections [78].

Notably, in renal biopsies of human FSGS and HIVAN, diminution in podocyte APOL1 expression preceded decreases in GLEPP1 and synaptopodin [78]. APOL1 appears to be constitutively. Some reports suggest that APOL1 sequesters phosphatidic acid and cardiolipin and promotes autophagocytic cell death [79]. Due to the importance of autophagy in modulation of podocyte aging [80], APOL1 may have substantial roles in podocyte homeostasis and survival. Importantly, APOL1 expression by cultured podocytes could also be induced by inflammatory mediators, such as tumor necrosis factor-α and lipopolysaccharide, indicating potential modulation by systemic influences [78].

Future studies focusing the clinical implications of APOL1 genotype in the setting of glomerulopathies and hypertension-associated kidney disease as well as exploring the cellular and molecular mechanisms of APOL1-associated

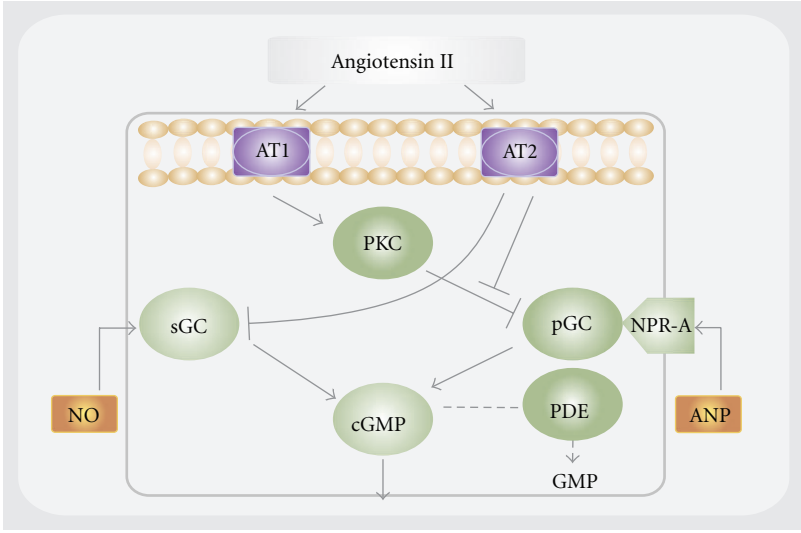


Figure 3: Schematic representation of the interaction between angiotensin II and guanylyl cyclase-dependent systems through AT1 and AT2 receptor in the podocyte. In podocytes, the guanylyl cyclase system may be modulated by angiotensin II via both AT1 receptor through pGC or AT2 receptor through sGC. In the absence of angiotensin II, both ANP and NO may also activate pGC and sGC, respectively. Abbreviations: cGMP: cyclic guanosine 5′-monophosphate; ANP: atrial natriuretic peptide; NO: nitric oxide; NPR-A: guanylyl cyclase-linked receptor for ANP; PKC: protein kinase C; sGC: soluble guanylyl cyclase; pGC: particulate guanylyl cyclase; PDE: phosphodiesterase. (Adapted from [81]).

disease may help to clarify these recent findings thus leading to new treatment approaches.

2.4. Podocytes and Vasoactive Molecules. In addition to forming a molecular glomerular filtration barrier, podocytes modulate filtration surface by counteracting the intracapillary pressure. The latter property is associated with a well-developed system of contractile proteins expressed in these cells as already mentioned above. Furthermore, podocytes not only express receptors for a number of vasoactive factors including atrial natriuretic peptide (ANP), nitric oxide (NO), and Ang II but are also capable of producing some of these hormones [81]. Additionally, podocytes present a number of second messenger systems including cyclic GMP and phospholipase C/inositol 1,4,5-triphosphate systems [82]. All these strongly suggest that at least some of the podocyte functions may be regulated by vasoactive substances.

Some experiments indicate that Ang II interacts with the systems generating vasoactive factors, either enhancing or antagonizing their activity [83–85]. Additionally, the modulatory effects of Ang II on the NO-dependent and ANP-dependent cGMP generation have also been reported [86–88]. In podocytes, Ang II has been shown to inhibit ANP-stimulated production of cGMP [89].

The synthesis of cGMP in cultured podocytes is modulated by Ang II via AT1 or AT2 receptors [82] (Figure 3).

2.5. Is the Glomerular Filtration Barrier Actually Efficient? How much protein crosses the glomerular barrier? Or in other words, how permeable is the glomerular barrier really? Undoubtedly, this last question has been a theme of major controversy for a long time. However, it is worth mentioning that most of the available information supports the idea that, in physiological conditions, the glomerular barrier is a functional structure with size and charge selectivity [90]. In contrast to this concept, some few studies [91, 92] postulate a glomerular sieving coefficient (GSC) for albumin of 0.034, which is much higher than the originally reported one (0.0006) [90]. Moreover, these experiments describe that renal albumin filtration in nonproteinuric rats is several times greater than previously measured filtrations and is followed by rapid endocytosis into proximal tubule cells. These findings suggest that the glomerular filter normally leaks albumin at nephrotic levels and that dysfunction of this retrieval pathway leads to proteinuria. Nevertheless, this hypothesis has been severely questioned on the light of the studies on megalin-cubilin, in which there is no retrieval of intact albumin. Filtered albumin binds to the megalin-cubilin complex of proximal tubule cells and is internalized, degraded, and released to blood as amino acids [93, 94]. Additionally, methodological objections to this so-called albumin retrieval hypothesis were also done after following physiological experiments using different techniques [95, 96]. Consequently, only modest amounts of albumin normally cross the barrier, but the amounts can increase greatly if there are barrier defects.

2.6. Proteinuria and Tubulointerstitial Response. In the last years, there has been an extensive debate on whether tubular albumin reabsorption and the subsequent lysosomal degradation may in fact result in tubular injury due to protein overload. The role of tubular reabsorption in urine protein homeostasis seems to be critical for the development of CKD [97]. During normal physiological conditions, all filtered

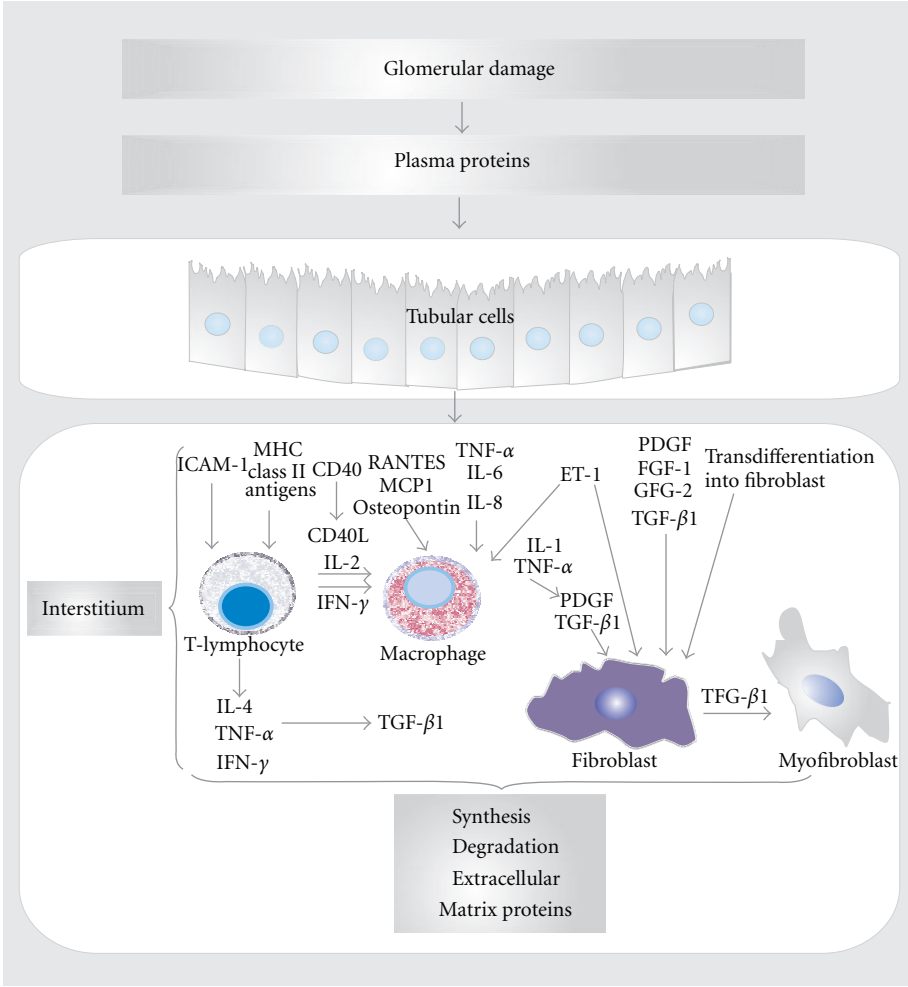


Figure 4: Molecules involved in potential mechanisms in the development of proteinuria-induced renal tubulointerstitial injury. The tubulointerstitial damage induced by persistent proteinuria in glomerular diseases is due to the concomitant injuring effects of multiple cellular and biochemical events. Specific proteins have been shown to stimulate production of cytokines, chemotactants, and matrix proteins by tubular epithelial cells, which may stimulate interstitial inflammation and scarring. Abbreviations: MHC: major histocompatibility complex; ICAM-1: intercellular adhesion molecule 1; MCP1: monocyte chemotactic protein-1; TNF-α: tumor necrosis factor-alpha; FGF: fibroblast growth factor; TGF-β1: transforming growth factor beta; PDGF: platelet-derived growth factor; ET-1: endothelin; RANTES: regulated upon activation, normal T-cell expressed and secreted; ECM: extracellular matrix. (Adapted from [99]).

proteins are efficiently internalized by the receptor complex megalin/cubilin/amnionless (AMN) by the proximal tubular epithelial cells, thus resulting in virtually protein-devoid urine [98]. In the proximal tubular cell, the proteins are degraded in lysosomes and substances such as vitamins are transported basally for reuse. However, in the case of glomerular injury, filtration of low-molecular-weight proteins increases and larger proteins start to penetrate the glomerular filtration barrier. Then, cells in the proximal tubule are thereby exposed to more, and new, proteins that overload the receptor-binding sites, thus leading to proteinuria. Furthermore, in the tubular cell, lysosomal degradation is unable to handle the increased amount of internalized protein, resulting in protein-clotted lysosomes. Although in most instances the primary event in triggering renal damage is glomerular injury, it is widely accepted that the severity in tubular and interstitial alterations is the driver for the development of fibrotic lesions, eventually resulting in ESRD.

Recent research has focused on how glomerular injury spreads to the tubulointerstitium, and currently, four possible mechanisms are being discussed: (1) obstruction of the urinary pole, (2) proteinuria-induced overload of the proximal tubule, (3) chronic hypoxia, and (4) inflammation induced by a glomerulotubular feedback loop. Figure 4 shows the diverse molecules involved in potential mechanisms in the development of renal tubulointestitial injury induced by proteinuria [99].

Therapeutic Approaches in Managing Patients with Proteinuria. It is widely accepted that the podocyte is the target of many types of injury mechanisms regardless of their nature. Hence, the loss of podocytes contributes to the development of glomerulosclerosis. Among them, the insult against

podocyte membrane antigens as in membranous nephropathy and minimal change disease and the consequence of hemodynamic injury produced by a reduced nephron number are both relevant causes linked to podocyte damage. Moreover, the already mentioned mutations in the genes of the glomerular filtration barrier-participating proteins (nephrin, laminin, TRPC6, α-actinin-4, CD2AP, type IV collagen, etc.) as well as the tremendous impact of metabolic disarrangements (especially diabetes and dyslipidemias) are associated with podocyte lesions [100–103]. Some other etiologies like protein overload states, drugs/toxins (NSADs, adriamycin), infectious diseases, and still unknown causes including idiopathic FSGS are also recognized causes of damage in glomerular visceral epithelial cell.

Undoubtedly, the inhibition of the renin-angiotensin-aldosterone system (RAAS) is associated with a maximum reduction in proteinuria and long-term renal risk reduction as well as a long-term renoprotection. Numerous clinical and experimental studies have demonstrated to reduce or control proteinuria by using either an angiotensin-converting enzyme inhibitor (ACEI) or angiotensin II receptor blocker (ARB) as a single therapy or in a dual blockade of RAAS [104–108]. However, due to individual risk factors, some patients present variable response to these therapeutic agents. It is worth emphasizing that dietary sodium restriction and diuretic therapy are similarly effective in improving the antiproteinuric action of the inhibition of RAAS. Therefore, the combination of both low-sodium diet and diuretic therapy may result in the highest reduction in proteinuria. Recently, direct renin inhibition has been included within the broad spectrum of RAAS blockade drugs. Aliskiren, a new direct renin inhibitor, reduced albuminuria significantly as it was demonstrated in a large study, in which proteinuric patients at the top of losartan treatment achieved a better control of albuminuria [109]. The blockade of aldosterone by either spironolactone or eplerenone together with and ACEI or ARB has been shown to have beneficial effects in patients with proteinuria, although the potential risk of hyperkalemia is increased [110, 111].

Although the interaction against the RAAS is the current therapeutic first line in patients with proteinuria, other drugs that may lower proteinuria independent of the RAAS action have become of great interest. In that sense and considering some pathophysiological mechanisms, new strategies such as vitamin D receptor activators and monocyte chemotactic protein-1 antagonists as well as endothelin antagonists emerge as potential alternatives in this setting. In addition, the combined therapy of these agents with the RAAS inhibition may potentiate antiproteinuric effects, thus exerting further renal protection.

In the field of new therapeutic perspectives in controlling glomerular damage, the VEGF antagonism emerges as a potential alternative. Based on the preliminary experimental studies in streptozotocin-diabetic rats with antibodies against VEGF in which renal function was improved [112], VEGF antagonism has been postulated as a promising tool in the treatment of diabetic nephropathy. Reinforcing this concept, nephropathy in db/db mice was attenuated by a small molecular inhibitor of angiogenesis [113] and by VEGF tyrosine kinase inhibitor [114]. However, tilting the balance against VEGF may cause harm. Chronic VEGF suppression may worsen interstitial fibrosis, and anti-VEGF antibody treatment was related to an exaggerated cystic response of the proximal tubules in cystic rats and severe kidney injury that was associated with low renal VEGF and high HIF-1α levels [115, 116]. Consequently, an extensive bulk of work is still needed to confidently assess the actually beneficial mechanisms and safety of VEGF antagonism for proteinuria. At present, anti-VEGF therapy remains as a double-edged sword in the nephrology scenario.

Taking into account the importance of the glomerular filtration barrier integrity, a more suitable therapeutic approach in order to stop or, at least, achieve a substantial reduction in proteinuria remains a big clinical challenge.

Disclosure

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Conflict of Interests

No conflicts of interest, financial or otherwise, are declared by the authors.

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Research Article

Serum Galactose-Deficient IgA1 Level Is Not Associated with Proteinuria in Children with IgA Nephropathy

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Introduction. Percentage of galactose-deficient IgA1 (Gd-IgA1) relative to total IgA in serum was recently reported to correlate with proteinuria at time of sampling and during follow-up for pediatric and adult patients with IgA nephropathy. We sought to determine whether this association exists in another cohort of pediatric patients with IgA nephropathy. *Methods*. Subjects were younger than 18 years at entry. Blood samples were collected on one or more occasions for determination of serum total IgA and Gd-IgA1. Gd-IgA1 was expressed as serum level and percent of total IgA. Urinary protein/creatinine ratio was calculated for random specimens. Spearman's correlation coefficients assessed the relationship between study variables. *Results*. The cohort had 29 Caucasians and 11 African-Americans with a male : female ratio of 1.9 : 1. Mean age at diagnosis was 11.7 ± 3.7 years. No statistically significant correlation was identified between serum total IgA, Gd-IgA1, or percent Gd-IgA1 versus urinary protein/creatinine ratio determined contemporaneously with biopsy or between average serum Gd-IgA1 or average percent Gd-IgA1 and time-average urinary protein/creatinine ratio. *Conclusion*. The magnitude of proteinuria in this cohort of pediatric patients with IgA nephropathy was influenced by factors other than Gd-IgA1 level, consistent with the proposed multi-hit pathogenetic pathways for this renal disease.

1. Introduction

IgA nephropathy (IgAN) is the most common form of chronic glomerulonephritis for individuals of European and Asian descent [1, 2]. The level of proteinuria at diagnosis of IgAN has been associated with the primary endpoint of outcome (i.e., progression to chronic dialysis or transplantation) in adults [3–7] and children [8–11].

Data from clinical and basic research in IgAN has led to the hypothesis that four hits are responsible for clinical expression of IgAN [12]. The first hit is the presence of aberrantly glycosylated *O*-linked glycans on the heavy-chain hinge region of circulatory IgA1 that terminate in *N*-acetylgalactosamine (GalNAc) rather than galactose [13]. Elevated serum levels of this galactose-deficient IgA1 (Gd-IgA1) were found in 76% of 153 Caucasian adults with IgAN in the United States [14]. IgAN patients in Japan and China also had elevated serum Gd-IgA1 levels [15, 16], as did African-American patients in the southeastern United States [17]. In addition, elevated serum Gd-IgA1 levels were found in 77%

of 22 African-American and Caucasian children with IgAN [18].

The second hit is the induction of circulating IgG or IgA antibodies specific for Gd-IgA1 [19], and the third hit is the resultant formation of nephritogenic immune complexes and their deposition in the glomerular mesangium. The final hit is the induction by these immune complexes of a local proliferative and inflammatory response of the mesangial cells [20–22]. Logically, the events related to the fourth hit would be directly or indirectly responsible for the induction of proteinuria in IgAN. In a recent study, the percentage of serum Gd-IgA1 relative to serum total IgA1 was found to correlate with proteinuria at time of sampling and over the follow-up interval in 62 pediatric and adult patients [23]. Those data differed from the findings at the time of sampling in our initial report with adult patients [14]. The aim of the present study was to determine whether the serum level of Gd-IgA1 associates with proteinuria in a well-characterized cohort of pediatric patients with IgAN.

2. Patients and Methods

2.1. Study Population. The subjects had been entered into earlier studies approved by the Institutional Review Boards of the University of Tennessee Health Science Center and the University of Alabama at Birmingham. The diagnosis of IgAN was established by renal biopsy showing IgA as the dominant or codominant immunoglobulin in a typical mesangial distribution, in the absence of clinical and laboratory evidence for systemic disease [24]. All subjects were younger than 18 years of age at the time of diagnostic biopsy. This study did not enroll children who had received a kidney transplant or who were on dialysis. Data for initial serum Gd-IgA1 level were previously reported for 22 of the patients included in the present report [17]. Of the 40 patients in this study, 31 were diagnosed and followed up by the pediatric nephrology group at the Le Bonheur Children's Hospital (LBCH) in Memphis, TN (LBCH cohort), five were diagnosed at other centers and seen in consultation at LBCH, two were diagnosed and followed in Lexington, KY, and two in Birmingham, AL.

Serum samples were available from 97 healthy controls younger than 18 years. The control group included 29 African-American males, 21 African-American females, 28 Caucasian males, and 19 Caucasian females.

2.2. Laboratory Measures and Data Collection. Blood samples were collected from patients on one or more occasions for determination of serum total IgA, Gd-IgA1, and creatinine concentration. Urinary protein and creatinine concentrations were measured in the clinical laboratory from a random spot urine sample, and a urinary protein/creatinine ratio (UPCR) (g/g) was calculated. Estimated GFR was calculated with the new Schwartz formula [25]. Systolic and diastolic blood pressure percentiles based upon age, gender, and height percentile were determined by the tables from the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents [26]. The blood pressure used for this calculation was the average of available measurements within two months of biopsy, if more than one was recorded. BMI percentile was determined using the QuesGen Systems, Inc. web-based calculator that used National Health and Nutrition Examination Survey data as the source for calculations (http://www.quesgen.com/BMIPedsCalc.php).

Time-average (TA) proteinuria was determined according to the description of Reich et al. [7], except that UPCR was used instead of results of timed urine collections. The UPCR was determined for each six-month interval after biopsy; if there were two or more values for an interval, the mean of the values was used. The TA-UPCR was derived by averaging these UPCRs from each six-month interval of follow-up time.

Serum total IgA and Gd-IgA1 levels were determined by ELISA, as described previously [14]. The Gd-IgA1 ELISA used biotinylated lectin from *Helix aspersa* (Sigma-Aldrich, St. Louis, MO, USA) that binds specifically to terminal GalNAc. Two galactose-deficient IgA1 myeloma proteins, McE and Ale, were used as standards in the Gd-IgA1 assays. Results for levels of Gd-IgA1 were expressed as U/mL serum, with 1 U (unit) corresponding to 1 μg of Gd-IgA1 myeloma standard protein. During the course of the study, the standard in the assay was changed from McE to Ale; the latter IgA1 myeloma protein has a slightly higher content of terminal GalNAc. The McE standard had been used exclusively for our initial reports [14, 17]. Subsequent levels determined using the Ale standard were multiplied by a factor of 2.5 to be compared to those determined in assays using the McE protein as standard.

2.3. Statistical Analyses. The Mann-Whitney *U* test was used to determine differences between patient and control groups for serum levels of Gd-IgA1 and percent Gd-IgA1/IgA. Spearman's correlation coefficients were used to assess the relationship between study variables. SAS 9.1 (SAS Institute, Cary, NC, USA) was used for descriptive statistics and calculation of correlation coefficients.

3. Results

Incident cases are defined as those having a serum Gd-IgA1 level first measured within 3 months after the diagnostic biopsy. Prevalent cases had their first Gd-IgA1 measurement after longer intervals. Clinical and demographic data are shown in Table 1 for the incident cases and in Table 2 for the prevalent cases.

The LBCH cohort differed from the other cases because of higher percentages of African-Americans and subjects with TA-UPCR determinations Table 3. The 40 patients included 29 Caucasians and 11 African Americans; 26 were male. For the IgAN group, the mean age ± SD at diagnosis was 11.7 ± 3.7 years and 13.2 ± 3.7 years at the time the first serum for Gd-IgA1 level was obtained. The mean ± SD age at time of study was 12.6 ± 2.9 years for the healthy control group.

Table 1: Clinical and demographic features of individual subjects (incident cases).

Subject	Race/gender	Presentation	Age at biopsy	estGFR (mL/min/1.73 m^2)	Urinary protein/ creatinine ratio	BMI percentile	SBP/DBP percentile	Serum Gd-IgA1 level (units/mL)
A1	C/M	Microhematuria, proteinuria	17.6	59	1.17	98	95–99/<50	262
M2	C/M	Isolated proteinuria	13.8	78	1.24	26	95–99/50–90	889
M15	C/F	Gross hematuria	11.2	80	1.31	<1	<50/50–90	1076
M16	C/F	Gross hematuria	8.7	75	1.00	57	50–90/<50	1073
M17	C/M	Gross hematuria	13.4	108	3.10	11	50–90/50–90	1534
M18	C/F	Recurrent gross hematuria	12.5	107	2.96	17	>99/<50	1155
M19	AA/F	Gross hematuria, rapidly progressive glomerulonephritis	15.9	49	2.97	52	<50, <50	3401
M21	AA/M	Isolated proteinuria	5.1	94	3.58	96	<50, 50–90	747
M22	C/M	Gross hematuria, acute kidney injury	8.6	75	1.33	49	95–99/50–90	803
M26	AA/M	Gross hematuria	8.8	120	3.70	30	90–95/50–90	470
M28	C/F	Gross hematuria, proteinuria	16.7	63	1.36	40	90–95/50–90	1110
M29	C/M	Gross hematuria	12.8	96	0.95	41	>99/95–99	659
M30	C/M	Gross hematuria, acute kidney injury	15.0	61	2.89	63	90–95/50–90	364
M31	C/M	Gross hematuria	16.2	84	0.25	89	50–90/50–90	2513
T2	C/M	Microhematuria, proteinuria, Chronic kidney disease stage 3	17.8	40	1.27	44	95–99/50–90	796

AA: African-American; BMI: body mass index; C: Caucasian; estGFR, estimated glomerular filtration rate; DBP: diastolic blood pressure; SBP: systolic blood pressure; microhematuria, >5 RBC/high powered field.

Measurements of serum Gd-IgA1 and UPCR on the same date were available on at least one occasion for 40 patients; 13 patients had from two to four paired samples over the course of observation. A total of 62 paired samples for serum Gd-IgA1 and UPCR were available for analysis.

The initial serum Gd-IgA1 levels for the patient and control groups are shown in Figure 1(a). The patient group levels were significantly higher than those of the control group ($P < 0.0001$). The median serum Gd-IgA1 level for 97 healthy controls under age 18 years was 260 U/mL (interquartile range (IQR) 183–334 U/mL). Based upon these controls, the 90th and 95th percentiles were 482 U/mL and 645 U/mL, respectively. For patients, median serum Gd-IgA1 level was 688 U/mL (IQR 517–1238 U/mL) and the median total serum IgA level was 2499 U/mL (IQR 1930–4072 U/mL).

The initial medians for percent Gd-IgA1/total serum IgA for the patient and control groups are shown in Figure 1(b). The patient group levels were significantly higher than those of the control group ($P < 0.0001$). The median percent Gd-IgA1/total serum IgA level for 97 healthy controls under age 18 years was 17%, with an IQR of 10% to 22%. Based upon these controls, the 90th and 95th percentiles were 32% and 34%, respectively. For patients, initial median percent Gd-IgA1/total serum IgA was 32% (IQR 26%–48%). The median UPCR was 0.74 g/g (IQR 0.23–1.68 g/g).

Spearman's correlation coefficients were also calculated for serum Gd-IgA1, percent Gd-IgA1, age at biopsy, and length of follow-up versus initial UPCR and TA-UPCR. For patients having two or more measurements for serum Gd-IgA1 and percent Gd-IgA1, the mean of these measurements was used for the calculation of correlation coefficients versus TA-UPCR. Again, all correlations were determined to be statistically insignificant and are as follows: serum Gd-IgA1 versus UPCR ($r = 0.05$, $P = 0.72$; Figure 2(a)), percent serum Gd-IgA1 versus UPCR ($r = -0.11$, $P = 0.38$; Figure 2(b)), serum Gd-IgA1 versus TA-UPCR ($r = 0.06$, $P = 0.75$; Figure 3(a)), percent serum Gd-IgA1 versus TA-UPCR ($r = 0.04$, $P = 0.87$; Figure 3(b)), age at time of biopsy versus TA-UPCR ($r = 0.18$, $P = 0.32$), and length of follow-up versus TA-UPCR ($r = 0.10$, $P = 0.60$). Of the 3 patients who progressed to ESRD, all had TA-UPCR above 1.0 and two had serum Gd-IgA1 levels above the 95th percentile for healthy children.

UPCR and serum Gd-IgA1 levels were plotted against age for the subject with the longest period of serial serum Gd-IgA1 sampling (Figure 4). This clinical course is of interest in that the blood sample for measurement of the first level was obtained during an episode of gross hematuria at time of diagnosis and the last three blood samples were collected during clinical remission (urinalysis, UPCR and serum creatinine concentration all normal).

Table 2: Clinical and demographic features of individual subjects (prevalent cases).

Subject	Race/gender	Presentation	Age at biopsy	estGFR (mL/min/1.73 m^2)	Urinary protein/ creatinine ratio	BMI percentile	SBP/DBP percentile	Serum Gd-IgA1 level (units/mL)
A2	AA/F	Gross hematuria, proteinuria	15.9	98	1.13	99	95–99/95–99	1320
A3	C/F	Gross hematuria, nephrotic syndrome	6.8	82	5.00	54		753
K1	C/M	Gross hematuria	11.2	87	1.40	99	50–90/50–90	704
K2	C/M	Microhematuria, proteinuria	13.2	138	0.41	99	>99/50–90	919
M1	AA/M	Microhematuria, proteinuria	9.3	108	300 mg/dL*	97	95–99/50–90	1328
M3	C/M	Isolated proteinuria, chronic kidney disease stage 3	13.0	50	4.12	95	95–99/50–90	594
M4	C/M	Recurrent gross hematuria, acute kidney injury	14.5	79	0.72	93	90–95/50–90	1867
M5	C/F	Gross hematuria	11.6	79	3.61	61	95–99/50–90	363
M6	C/M	Gross hematuria	11.6	98	255 mg**	74	90–95/95–99	2177
M7	AA/F	Gross hematuria, nephrotic syndrome, acute kidney injury	10.4	70	3.29	33	50–90/50–90	315
M8	C/M	Gross hematuria	17.4	67	0.44	94	90–95/50–90	1054
M9	C/M	Recurrent gross hematuria	16.5	100	0.41	76	90–95/50–90	861
M10	AA/M	Recurrent gross hematuria	7.0	102	0.64	90	95–99/50–90	563
M11	AA/F	Gross hematuria	5.5	95	30 mg/dL*	93	50–90/<50	882
M12	C/M	Gross hematuria	8.5	87	0.04	15	50–90/50–90	612

AA: African-American; BMI: body mass index; C: Caucasian; estGFR: estimated glomerular filtration rate; DBP: diastolic blood pressure; SBP: systolic blood pressure.
*Amount of protein by urinary dipstick;
**Amount of protein in 24 hours.

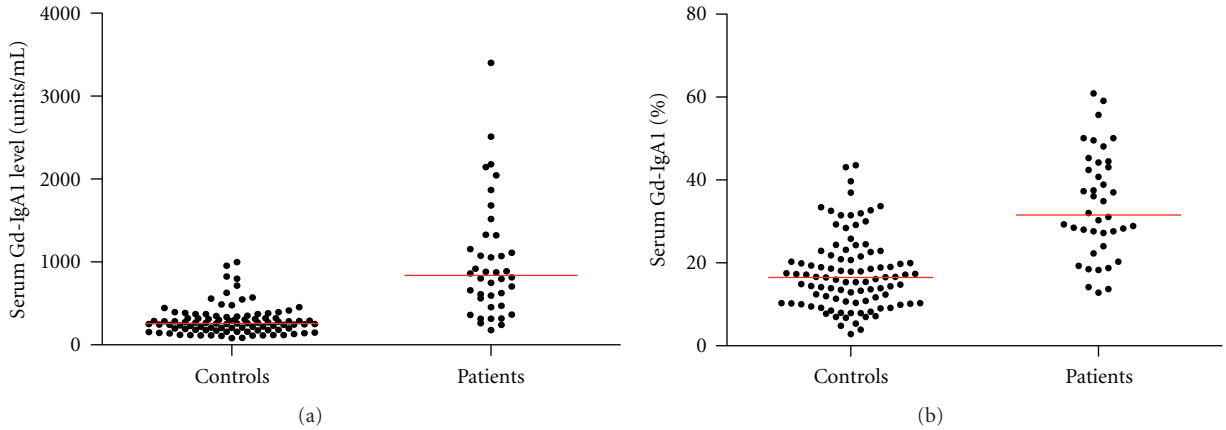

Figure 1: Serum levels of Gd-IgA1 plotted for the initial sample for 40 pediatric patients and 97 healthy pediatric controls. (a) Represented as units/mL serum. Median is represented for each group. The serum level was significantly higher for the patient group ($P < 0.0001$). (b) Represented as percentage of total IgA. Median is represented for each group by the red bar. The serum level was significantly higher for the patient group ($P < 0.0001$).

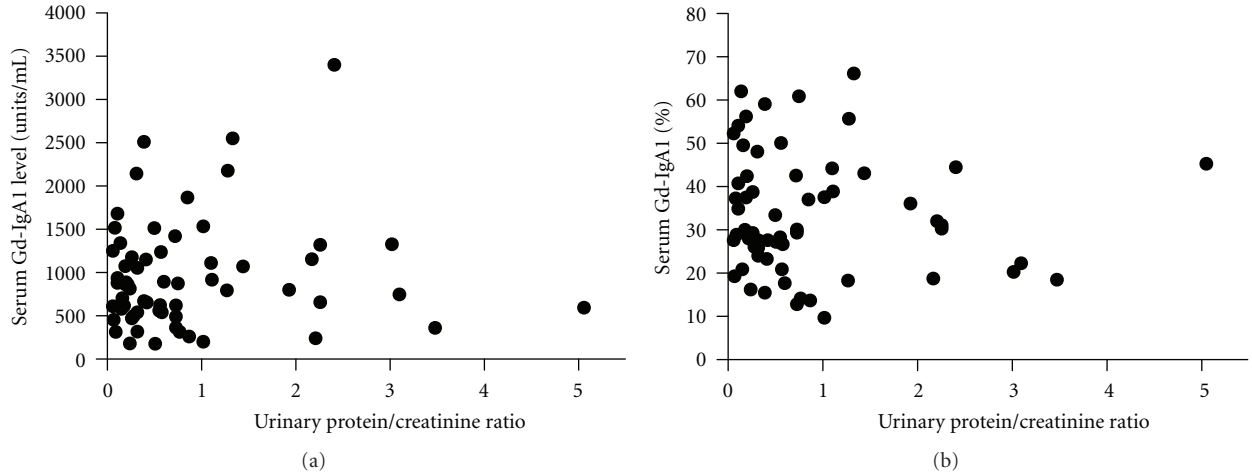

Figure 2: Random urinary protein/creatinine ratios. (a) Plotted against serum Gd-IgA1 levels. Spearman's rank correlation is $r = 0.05$, $P = 0.72$. (b) Plotted against percent Gd-IgA1 levels. Spearman's rank correlation is $r = -0.11$, $P = 0.38$.

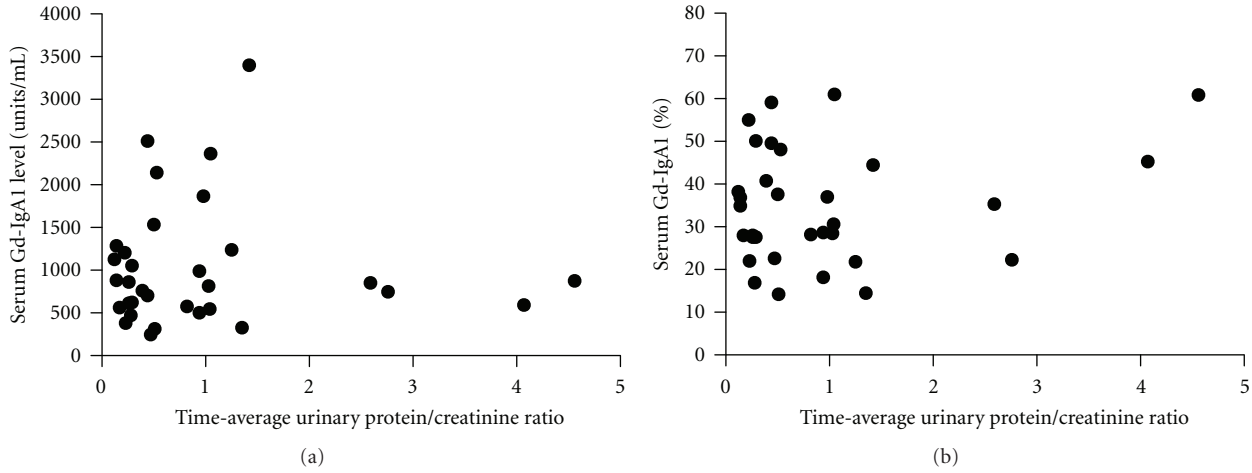

Figure 3: Time-average urinary protein/creatinine ratios. (a) Plotted against average serum Gd-IgA1 levels. Spearman's rank correlation is $r = 0.06$, $P = 0.75$. (b) Plotted against serum percent Gd-IgA1 levels. Spearman's rank correlation is $r = 0.04$, $P = 0.87$.

4. Discussion

Remission of proteinuria is an important predictor of renal survival. Analysis of 542 adult patients with IgAN in the Toronto Glomerulonephritis Registry showed that when treatment achieved a mean urinary protein excretion <1 g/day over the follow-up interval, the decline in glomerular filtration rate was markedly slower than that for the entire cohort [7]. Thus, magnitude of proteinuria is a useful surrogate marker of outcome in IgAN patients detected early in the course of disease when renal clearance function is normal.

Serum Gd-IgA1 level can be expressed as an absolute level or as a percentage of total serum IgA. In our earlier cohort of 153 adults with IgAN, the absolute serum level was a better diagnostic marker than percent Gd-IgA1, but neither Gd-IgA1 ($r = -0.128$, $P = 0.211$) [14] nor percent Gd-IgA1 ($r = -0.022$, $P = 0.788$) (previously unpublished data) correlated significantly with UPCR. In that study, only 24 subjects were sampled within 8 weeks of biopsy for measurement of the serum Gd-IgA1 level [14]. However, the median serum Gd-IgA1 level for those 24 subjects was similar to the median for 26 subjects who had levels measured at the last follow-up when the urinalysis and UPCR were normal.

In a Japanese cohort, however, there was no clinically significant difference in magnitude of proteinuria when subjects over the age of 16 years were stratified by serum Gd-IgA1 level above versus below the 90th percentile for healthy controls [15]. The urine protein excretion for the 20 subjects with serum Gd-IgA1 levels above the 90th percentile was 1.0 g/day versus 1.1 g/day for the 21 subjects with lower serum Gd-IgA1 levels. About 95% of the serum samples used for that study were obtained at the time of biopsy.

In a study by Camilla et al. [23], their cohort included adults and children with IgAN and a correlation between percent Gd-IgA1/IgA and contemporaneous UPCR was

Table 3: Clinical and demographic features of cohorts.

	LBCH cohort $n = 31$	Other Cases $n = 9$
Incident cases	13	2
Prevalent cases	18	7
Male	21	5
Female	10	4
Caucasian	21	8
African-American	10	1
Age at biopsy, yrs	11.5 ± 4.7	12.4 ± 4.9
Follow-up after biopsy all patients, yrs, mean ± SD	4.2 ± 2.4	1.9 ± 2.0
CKD5 at last follow-up	3	0
TA-UPCR data, patients	29	3
Follow-up after biopsy for TA-UPCR patients, yr, mean ± SD	4.2 ± 2.4	3.1 ± 2.1
TA-UPCR ≥ 1.0	9	1
TA-UPCR ≥ 0.5, < 1.0	7	0
TA-UPCR < 0.5	13	2

CKD5: chronic kidney disease stage 5; LBCH: Le Bonheur Children's Hospital; SD: standard deviation; TA-UPCR: time-average urinary protein/creatinine ratio; yrs: years.

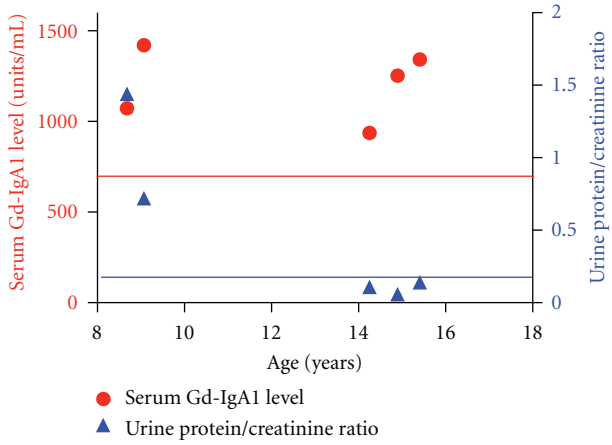

Figure 4: Serum Gd-IgA1 levels (red circles) and concomitant urinary protein/creatinine ratios are plotted for the patient having the most serum Gd-IgA1 determinations. The red line represents the 95th percentile for serum Gd-IgA level in healthy pediatric controls, and the blue line represents the upper limit of normal for urinary protein/creatinine ratio of 0.2. This time course is of interest in that the initial value was at presentation with gross hematuria and the last three values were obtained during clinical remission (normal urinalysis and estimated GFR).

found to be marginally significant ($r = 0.25$, $P = 0.03$). The correlation improved when TA-UPCR for each patient was correlated against a single serum Gd-IgA1 level ($r = 0.29$, $P = 0.007$). Our failure to confirm this finding in a pediatric cohort is not easily explained. A possible basis is a difference in severity of disease. Our pediatric cohort had a higher percentage of patients with low TA-proteinuria.

Serum Gd-IgA1 and percent Gd-IgA1 levels are often elevated in first-degree relatives of patients with IgAN [15, 27, 28]. However, virtually all of these affected relatives had no clinical evidence for renal disease prior to or at the time of blood sampling [15, 27, 28]. In addition, careful examination of the values for healthy controls indicates that serum Gd-IgA1 levels are distributed in a nonparametric manner, such that at least 5% of the subjects had a level higher than predicted by a normal distribution [14, 15, 23].

Thus, the presence of serum Gd-IgA1 is necessary but not sufficient to precipitate the renal injury in IgAN [12]. In the study of Suzuki et al. [19], levels of IgG specific for Gd-IgA1 correlated with UPCR ($P < 0.0001$) and with the levels of IgA-IgG immune complexes normalized to urinary creatinine ($P = 0.0082$) in contemporaneously collected urine samples. This prognostic significance may be due to a requirement for the formation of immune complexes to sustain renal injury in IgAN. The serum Gd-IgA1 level seems to be more valuable as a marker for risk of disease rather than for prognosis [14].

5. Conclusion

In a cohort of pediatric patients with IgAN, we failed to confirm a recently described association between the magnitude of proteinuria and percent serum Gd-IgA1/IgA [21]. This finding is similar to that in our initial North American cohort and a Japanese cohort of adults [14, 15]. Thus, the weight of the evidence to date fails to support a relationship between the serum Gd-IgA1 level and severity of proteinuria. This clinical expression of disease in IgAN is likely influenced by other factors or hits, such as levels of circulating antibodies specific for Gd-IgA1, level and/or composition of Gd-IgA1-containing immune complexes, or other factors that influence mesangial inflammation.

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Review Article

Is the Antiproteinuric Effect of Cyclosporine A Independent of Its Immunosuppressive Function in T Cells?

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The antiproteinuric effect of cyclosporine A(CsA) has been believed to result from its immunosuppressive effect on the transcription factor NFAT in T cells. However, current evidences supporting this hypothesis are missing. A recent study showed that CsA has a direct antiproteinuric effect on podocytes, suggesting a novel non-immunosuppressive mechanism for CsA's antiproteinuric effect. Conditional NFATc1 activation in podoyctes per se is sufficient to induce proteinuria in mice, indicating that NFAT activation in podocytes is a critical pathogenic molecular event leading to podocyte injury and proteinuria. Meanwhile, evidence showed that TRPC6-mediated Ca^{2+} influx stimulates NFAT-dependent TRPC6 expression. Altogether, these advances in podocyte research indicate that calcineurin-NFAT signal or calcineurin-synaptopodin axis has a direct proteinuric effect on podocytes which raises the possibility of developing specific antiproteinuric drugs that lack the unwanted effects of calcineurin or NFAT inhibition.

1. Introduction

Clinically, calcineurin inhibitors (e.g., cyclosporine A, CsA) have been used to reduce proteinuria in focal segmental glomerulosclerosis (FSGS), minimal change disease (MCD), and other proteinuric kidney diseases [1]. T-cell dysfunction is associated with some forms of proteinuria, including a subset of MCD in children. This concept originally stemmed from the so called "Shalhoub hypothesis" that "lipoid nephrosis" is produced by a systemic abnormality of T-cell function [2]. For decades, FSGS was thought to be an immunologic disease resulting from the noxious effect of a lymphokine on the podocyte [3]. This was the primary reason for using CsA as an immunosuppressive drug, to continue to endorse this mechanism of action despite studies demonstrating that calcineurin inhibition reduced proteinuria in nonimmunologic glomerulopathies. Actually, CsA can also reduce proteinuria in human and experimental Alport's syndrome, a nonimmunological disease, raising doubts of this hypothesis [4, 5]. Moreover, although CsA effectively lowered the level of proteinuria in human membranous nephropathy, the study of Ambalavanan et al. showed a more numerous and lager electron-dense immune deposits than before CsA's therapy, indicating that whilst CsA exerts an antiproteinuric effect, the drug does not modify the histologic aggravation of the glomerular lesions [6]. A recent study showed that CsA has a direct antiproteinuric effect on podocytes [7].CsA blocked calcineurin-mediated dephosphorylation of the actin-organizing protein synaptopodin, a podocyte foot process cytoskeletal component, facilitating its degradation by Cathespin L. Mechanistically, this study thus identified a new calcineurin signaling pathway in kidney podocytes and attributed the antiproteinuric effect of CsA to its inhibition of calcineurin-mediated degradation of synaptopodin. Thus, this antiproteinuric effect was shown to be independent of T cells, at least partially. Although arguing against an antiproteinuric role of CsA through the suppression of T cells, this study did not rule out the involvement of NFAT proteins downstream of calcineurin in kidney podocytes. Our results, along with those from Wang et al. [8] and Nijenhuis et al. [9], suggested that conditional NFATc1 activation in podocytes per se is sufficient to induce

proteinuria in mice. Thus, both studies provided in vivo evidence that NFAT activation in podocytes may be a critical pathogenic molecular event leading to proteinuria or FSGS. Altogether, these advances in podocyte research indicate that calcineurin-NFAT signal or calcineurin-synaptopodin axis has a direct proteinuric effect on podocytes, and these observations raise the possibility of developing specific antiproteinuric drugs that lack the unwanted effects of calcineurin or NFAT inhibition [10].

2. Proteinuria and Its Molecular Mechanisms in Podocyte

Proteinuria, a cardinal sign and a prognostic marker of kidney disease, affects several hundred million people worldwide [12]. Proteinuria is also an independent risk factor for cardiovascular morbidity and mortality. Podocytes, endothelial cells, and the glomerular basement membrane (GBM) constitute the glomerular filtration barrier, a highly specialized structure for selective ultrafiltration. The common denominator in a variety of kidney diseases, including MCD and FSGS, is podocyte injury involving a massive loss of protein in the urine (proteinuria) [13, 14]. Several studies showed that the podocyte has a central role in the development of proteinuria and idiopathic nephrotic syndrome [14]. Effacement of the podocyte foot processes is a common feature of proteinuric diseases [15]. Several pathogenic pathways involved in effacement of the podocyte foot processes and the development of proteinuria have been discovered. Studies in hereditary proteinuric syndromes have uncovered that mutations of podocyte proteins, including α-actinin-4 [16], CD2AP [16, 17], nephrin [18], PLCE1 [19], podocin [20], TRPC 6 [21, 22], formin protein INF2 [23], and MYO1E [24] lead to proteinuria, podocyte foot processes effacement and podocyte actin cytoskeleton disruption [14, 25]. Other proteins regulate the podocyte actin cytoskeleton and are important for the glomerular filtration barrier [25]. These proteins include Rho GDIalpha [26, 27], podocalyxin [28], FAT1 [29], Nck1/2 [30], and synaptopodin [31].

TRPC6 is a member of the large transient receptor potential superfamily of nonselective cation channels [32, 33]. Mutations in the gene that encodes TRPC6 have been identified in families with autosomal-dominant FSGS [34–36]. Many calcium-dependent signals, including calcineurin, may be potential targets of TRPC6 activation in podocytes since TRPC6 are involved in the regulation of intracellular calcium concentration in response to the activation of G-protein-coupled receptors and receptor tyrosine kinases. Meanwhile, some TRPC6 mutations found in human beings with FSGS result in increased amplitude and duration of calcium influx into HeK293 cells [21, 22]. TRPC6-deficient mice do not show any obvious renal phenotype [34]. However, transient induction of TRPC6 overexpression by in vivo gene delivery results in proteinuria [33].

The actin-binding protein synaptopodin, which is highly expressed in podocytes [35], is a key regulator of podocyte function since bigenic heterozygosity for synaptopodin and CD2-associated protein results in proteinuria and FSGS [37]. Synaptopodin induces stress fibers by stabilizing the GTPase RhoA [31] and suppresses filopodia by disrupting cell-division cycle-42-insulin receptor substrate p53-Mena signaling complexes [38].

Recently, urokinase receptor (uPAR) and its soluble form (suPAR) have been shown to be involved in the pathogenesis of proteinuria and FSGS [39, 40]. uPAR is a glycosylphosphatidylinositol-anchored protein that has been shown to be a proteinase receptor for urokinase but has also been involved in nonproteolytic pathways, mainly through interactions with other plasma membrane proteins such as integrins [41]. uPAR and β3 integrin colocalize in podocytes and thus form a complex with β3 integrin, thereby causing the activation of β3 integrin. In vivo gene delivery of constitutively active β3 integrin is sufficient to induce proteinuria in mice; conversely, inhibition of uPAR expression and β3 integrin activation has an antiproteinuric effect [42].

Interestingly, suPAR, a soluble form of uPAR has been identified as a circulating FSGS factor that is elevated in the serum of approximately two-thirds of primary FSGS patients. suPAR-mediated activation of β3 integrin on podocyte foot processes may be the mechanism of injury caused by high suPAR blood concentrations [40].

3. The Role of Calcineurin Signal in Podocyte Injury and Proteinuria

The calcineurin is ubiquitously expressed in all mammalian tissues and is a Ca^{2+}-dependent serine/threonine phosphatase composed of a catalytic subunit, CnA, and a regulatory subunit, CnB [43, 44]. Calcineurin has a wide range of roles in organ development and cellular functions [45, 46], including the regulation of transcription in various renal cells [43, 44].

Clinically, CsA is used to reduce proteinuria in kidney diseases, such as idiopathic nephrotic syndrome (especially FSGS). The immunosuppressive effect of CsA results from inhibition of signaling by the transcription factor NFAT in T cells [47], and this action has also been believed to mediate CsA's antiproteinuric effect. Although the efficacy of CsA was thought to derive from its suppression of NFAT activation in T cells through the inhibition of calcineurin, not all drugs suppressing T-cell activation have protective effects on glomeruli [1]. The study of Faul et al. [7] suggested a novel role for CsA in inhibiting the dephosphorylation of synaptopodin by calcineurin. Faul et al. [7] found that activation of calcineurin in the podocyte is sufficient to cause proteinuria via the degradation of synaptopodin and that CsA blocks the calcineurin-mediated dephosphorylation of synaptopodin, thereby preserving the phosphorylation-dependent synaptopodin-14-3-3b interaction. They also identified synaptopodin as a substrate of PKA and CaMKII. CsA and E64 ameliorate LPS-induced proteinuria by blocking the CatL-mediated degradation of synaptopodin [48–51]. These data unveiled a calcineurin signaling pathway,

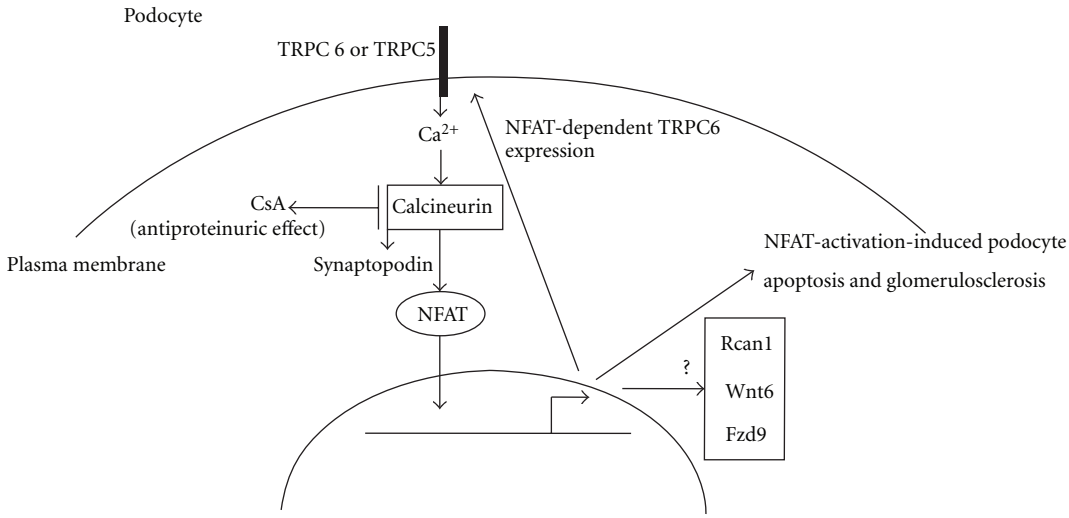

Figure 1: The calcineurin-activation-induced podocyte injury. Upon activation by increased intracellular Ca^{2+}, calcineurin dephosphorylates the NFAT proteins and synaptopodin [5, 6]. NFAT activation induces podocyte apoptosis [11] and glomerulosclerosis [6]. TRPC6-mediated Ca^{2+} influx stimulates NFAT-dependent TRPC6 expression [7]. Rcan1, Wnt6, and Fzd9 were shown to be upregulated in glomeruli isolated from $NFATc1^{nuc}$ transgenic mice, making them potentially direct targets of NFAT [6].

which is operative in podocytes and contributes to the maintenance of kidney filter function.

Although Faul et al. argued against an antiproteinuric role of CsA through the suppression of T cells, it is possible for the involvement of NFAT proteins downstream of calcineurin in podocyte injury, since the NFAT transcription factors are the most extensively studied calcineurin substrates and the major regulators of transcription in response to Ca^{2+}/calcineurin signals [47, 52].

Ca^{2+} signaling through ion channels has recently emerged as a potential modulator of podocyte function, and several Ca^{2+}-permeable channels have been identified inpodocytes [53, 54]. Generally, upon activation by increased intracellular Ca^{2+}, calcineurin dephosphorylates the NFAT proteins that reside in the cytoplasm in resting cells. This dephosphorylation exposes the concealed nuclear localization signals of the NFAT proteins, leading to the cytoplasm to nucleus translocation of these proteins. In the nucleus, the NFATc proteins form NFAT transcription complex with their nuclear partners to control the transcription of target genes.

TRPC6 mutations were found in families with hereditary FSGS, and TRPC5 and TRPC6 channels are now known as the Ca^{2+} influx pathways for the nonselective, cationic current in podocytes [55]. Mutations in one of these channels,TRPC6, lead to aberrant Ca^{2+} signaling, podocyte dysfunction [21, 22], and Nephrin and Neph1 have been shown to interact with several Ca^{2+} channels, including TRPC6 [11, 56]. Vassiliadis et al. [57] showed that Ca^{2+}/calcineurin signals mediated podocyte injury. Inhibition of calcium channels and chelation of extracellular calcium reduced protamine sulfate-induced damage, suggesting that calcium signaling plays a critical role in the initial stages of glomerular injury. Calcineurin inhibitors (FK506 and CsA) inhibited protamine sulfate-mediated barrier changes, indicating that calcium signaling acts, in part, through calcineurin-dependent cleavage of synaptopodin. Meanwhile, mutations in TRPC6 enhance the amplitude and duration of the Ca^{2+} channel current which cause NFAT activation, indicating the activation of the calcineurin-NFAT pathway as a potential mediator of FSGS [58].

Importantly, the study of Wang et al. [8] demonstrated that, in parallel to synaptopodin regulation, there may be an additional pathway from calcineurin to podocyte injury and proteinuria that involves NFAT-mediated regulation of known and novel factors important for podocyte function. To study the role of NFAT signaling in glomerular podocytes, Wang et al. [8] created a system for inducible activation of NFAT signaling in podocytes, in which a Podocin-Cre transgene was used to induce the removal of the transcriptional stop cassette in a ROSA26-rtTA allele only in podocytes [59, 60]. And when the cassette is deleted, the ROSA26 promoter drives the production of reverse tetracycline-controlled transactivator (rtTA) in podocytes. When treated with doxycycline, the doxycycline-rtTA complex binds to the *TetO* sequence of the $TetO\text{-}NFATc1^{NUC}$ transgene. The mice carrying three alleles (*Podocin-Cre, RO-SA26-rtTA, and* $TetO\text{-}NFATc1^{NUC}$) were referred as mutants. Thus, results from Wang et al. [8] provided in vivo evidence that NFAT activation, either in utero or postdevelopmentally, can lead to podocyte injury and proteinuria, which suggest that activation of NFAT signaling may be a key pathogenic molecular change in podocyte injury and the development of proteinuria.

Using a similar model for conditional NFAT activation in podocytes, Nijenhuis et al. [9] demonstrated that podocyte-specific inducible expression of a constitutively active NFAT mutant increased TRPC6 expression and induced severe proteinuria, and that calcineurin inhibition by CsA

downregulated TRPC6 expression and reduced proteinuria. Importantly, this study showed that a deleterious feed-forward mechanism, in which TRPC6-mediated Ca^{2+} influx stimulates NFAT-dependent TRPC6 expression, is involved in angiotensin II (Ang II)-associated podocyte injury. In vitro and in vivo models, Ang II, a key contributor to the pathogenesis of glomerular disease, increases TRPC6 expression in podocytes. The regulation of TRPC6 expression by AngII is dependent on TRPC6-mediated Ca^{2+} influx and the activation of the Ca^{2+}-dependent calcineurin/NFAT signaling. Tian et al. [61] showed that Ang II resulted in significant reduction in the abundance of synaptopodin, and gene silencing of TRPC6 resulted in loss of synaptopodin in podocytes in contrast, gene silencing of TRPC5 did not affect synaptopodin abundance. TRPC6-depleted cells treated with CsA restored synaptopodin abundance, suggesting an association between Ca^{2+} influx through TRPC5 and TRPC6 channels and synaptopodin signaling in podocytes. Schlöndorff et al. [58] showed that that all three TRPC6 mutations (P112Q, R895C, and E897K) to enhance channel activity lead to enhanced basal NFAT-mediated transcription in cultured podocytes, which are dependent on channel activity and are dominant when mutants are coexpressed with wild-type TRPC6. Activation of NFAT by TRPC6 mutants is blocked by inhibitors of calcineurin, calmodulin-dependent kinase II, and phosphatidylinositol 3-kinase.

In addition, another study showed that calcineurin induces podocyte apoptosis in a genetic model of type 1 diabetes mellitus (Akita mice). In cultured podocytes, activated NFAT promotes podocyte apoptosis in a calcineurin-dependent fashion, and induction of apoptosis by either angiotensin II or endothelin-1 was blocked by a calcineurin inhibitor (FK506). This induction of apoptosis appears to require NFAT-induced gene transcription [62].

In other cell populations, NFATc1 activation leads to distinctive changes in transcription and cellular behavior [63–65]. Interestingly, Rcan1, Wnt6, and Fzd9 were shown to be upregulated in glomeruli isolated from *NFATc*1^{nuc} transgenic mice, making them potentially direct targets of NFATc1[8]. The upregulation of Rcan1, a known target of NFAT [66, 67], may reflect a potential regulation axis of calcineurin, NFAT, and Rcan1 in podocyte. Moreover, upregulation of Wnt signaling was found to be detrimental to podocytes [68], and Wnt signaling was also shown to be upregulated in *NFATc*1^{nuc} transgenic mice, indicating that the upregulation of Wnt signaling seems to be the pathogenesis of NFAT activation-induced podocyte injury and FSGS.

4. Conclusion

The antiproteinuric effect of CsA is attributed to its immunosuppressive effect. However, recently published researches showed that the effect of CsA on proteinuria is not dependent on NFAT inhibition in T cells, but rather results from its effects on kidney podocytes, including the stabilization of synaptopodin. Moreover, in vivo evidence from *NFATc*1^{nuc} transgenic mice and Akita mice showed NFAT activation-induced podocyte apoptosis and injury, and NFAT-dependent TRPC6 expression, the mutations of which have been shown to be associated with proteinuria and glomerulosclerosis in human patients. In summary, recently, there is the possibility that CsA might have a coupled nonimmunological antiproteinuric effect: one as demonstrated by Faul et al. on synaptopodin and the podocyte cytoskeleton, another separate one through the inhibition of NFAT as NFAT induces proteinuria and podocyte apoptosis (Figure 1).

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Review Article

Progress in Pathogenesis of Proteinuria

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Aims. Proteinuria not only is a sign of kidney damage, but also is involved in the progression of renal diseases as an independent pathologic factor. Clinically, glomerular proteinuria is most commonly observed, which relates to structural and functional anomalies in the glomerular filtration barrier. The aim of this paper was to describe the pathogenesis of glomerular proteinuria. *Data Sources*. Articles on glomerular proteinuria retrieved from Pubmed and MEDLINE in the recent 5 years were reviewed. *Results*. The new understanding of the roles of glomerular endothelial cells and the glomerular basement membrane (GBM) in the pathogenesis of glomerular proteinuria was gained. The close relationships of slit diaphragm (SD) molecules such as nephrin, podocin, CD2-associated protein (CD2AP), a-actinin-4, transient receptor potential cation channel 6 (TRPC6), Densin and membrane-associated guanylate kinase inverted 1 (MAGI-1), $\alpha 3\beta 1$ integrin, WT1, phospholipase C epsilon-1 (PLCE1), Lmx1b, and MYH9, and mitochondrial disorders and circulating factors in the pathogenesis of glomerular proteinuria were also gradually discovered. *Conclusion*. Renal proteinuria is a manifestation of glomerular filtration barrier dysfunction. Not only glomerular endothelial cells and GBM, but also the glomerular podocytes and their SDs play an important role in the pathogenesis of glomerular proteinuria.

1. Introduction

Under normal conditions, high molecular weight proteins in the plasma (e.g., albumin and globulin) cannot pass through the filtration membrane due to the effects of the size barrier and charge barrier of the glomerular capillary filtration membrane. Low molecular weight proteins (e.g., β2-microglobulin (β2-M), α1-microglobulin (α1-M), and lysozyme), however, can freely pass through the filtration membrane, although the filtration amount is low and 95% of these proteins are reabsorbed when entering the proximal convoluted tubule. The final urine protein content is therefore low (only 30–130 mg/24 h) and consists primarily of plasma albumin (40%), immunoglobulin fragments (15%), other plasma proteins (5%), and urinary system-originating tissue proteins (40%). The protein concentration in a random urine sample is 0–80 mg/L, and the results of qualitative tests for urokinase protein are typically negative. When the urine protein exceeds 150 mg/24 h or the concentration is above 100 mg/L, the result for the qualitative protein test becomes positive. This is known as proteinuria [1, 2].

Proteinuria is the most common manifestation of renal diseases. Reviews of a number of experimental studies have shown that proteinuria is not only a sign of kidney damage, but also participates in the progression of renal diseases as an independent pathologic factor [3–5]. Clinically, glomerular proteinuria is most commonly observed and relates to structural and functional anomalies in the glomerular filtration barrier. In recent years, researchers have not only gained a new understanding of the roles of glomerular endothelial cells and the glomerular basement membrane (GBM) in the pathogenesis of proteinuria, but have also gradually discovered the close relationships of slit diaphragm (SD) molecules such as nephrin, podocin, CD2-associated protein (CD2AP), a-actinin-4, transient receptor potential cation channel 6 (TRPC6), Densin, and membrane-associated guanylate kinase inverted 1 (MAGI-1) in the pathogenesis of proteinuria. In this paper, research progress in the pathogenesis of glomerular proteinuria in recent years is reviewed.

2. The Structure of the Glomerular Filtration Barrier

The glomerular filtration barrier is comprised of three layers. (1) Capillary endothelial cells in the inner layer. A variety of fenestrae, small ostioles of 50–100 nm in diameter on endothelial cells, may prevent hematocytes from passing through. These fenestrae may not intercept plasma proteins to be filtrated, but the negative proteoglycans on their surface may exert some charge barrier effect. (2) The cellular basement membrane in the interface layer. This is the major filtration barrier of the filtration membrane, is approximately 100 nm thick, and consists of the inner and outer loose tectoria and the intermediate stratum compactum. The GBM is a microfibrous reticulum structure composed of hydrated gel that exerts its charge-barrier and size-barrier functions via its rich surface anion charges and fibrous strap meshwork-like aperture screen stencil. (3) The visceral epithelial cells in the outer layer. Epithelial cells have podocytic processes that form fissures among them through reciprocal overlapping. There is a layer of filtration fissure membrane on the fissures with holes of 4–14 nm in diameter on its surface that represent the last barrier of the filtration membrane. Under normal circumstances, the radius of an effective filtration hole in the glomerular filtration membrane is approximately 30 angstroms. Small molecular proteins such as lysozyme and β2-microglobulin can be filtered, while proteins with molecular weights above 60,000–70,000 are difficult to filter. The molecular weight of plasma albumin is 69,000, and the molecular radius is approximately 37 angstroms, making it difficult to pass through the filtration membrane. Pathologically, as a result of antibasement membrane antibody and immune complex deposition, as well as the release of cytokines and inflammatory mediators, glomerular capillary damage may occur with increased permeability. This allows a large number of plasma proteins, especially albumins in the Bowman's capsule, to exceed the proximal renal tubule's ability to reabsorb proteins and induce proteinuria.

3. Glomerular Endothelial Cells and Proteinuria

The difference between glomerular endothelial cells and other vascular endothelial cells lies in their flatter surfaces and fenestrae that are approximately 50–100 nm in diameter. Glomerular endothelial cells are the first line of defense of the glomerular filtration barrier. The fibrils, which are 7 nm thick and exist inside the endothelial cytoplasm, constitute the fenestrate structure. Changes in the aperture of fibrils may affect vessel wall permeability. Endothelial cell structural proteins (e.g., actin, myoglobulin) may also affect the diameter of the fenestrate structure via growth factors. In addition, synergism between the inherent special structures (such as cell membrane-like depression, zonula occludens, and glycocalyx) of glomerular endothelial cells and circulatory permeability factors (such as a-acidic mucin, apolipoprotein, and Amadori's product) may generate the endothelial cell-GBM-podocyte axis, thus playing a special role in maintaining the integrity of the filtration barrier [6, 7].

The cell coat on the surface of glomerular endothelial cells, also known as glycocalyx, is approximately 300 nm thick. It consists of proteoglycan, glycosaminoglycan, and plasma proteins such as orosomucoids that are rich in negative charges. Plasma proteins can be secreted by endothelial cells and are closely related to the permeability of glomerular capillaries. Glycocalyx can affect blood flow velocity and exert a selective barrier effect on macromolecular movement. Damage to systemic endothelial glycocalyx is associated with the onset of albuminuria in patients with type 1 diabetes [8]. Glycocalyx is perturbed in individuals with type 2 diabetes, and oral glycocalyx precursor treatment improves glycocalyx properties [9]. Recently, it has been reported that damage to the endothelial glycocalyx alters the permeability of multiple capillary beds: in the glomerulus this is clinically apparent as albuminuria [10]. It has been found in endothelial cells cultured in vitro that the glycosaminoglycan hyaluronan in glycocalyx may generate a matrix with molecular sieve properties in order to maintain the charge barrier [11, 12]. In addition, orosomucoid plays an important role in maintaining the charge barrier by interacting with the glycocalyx of endothelial cells [6, 13].

4. GBM and Proteinuria

Like the basement membranes of other structures of the human body, the GBM is a microfibrous reticular structure primarily consisting of collagen type IV, laminin (primarily laminin-11 and $\alpha5\beta2\gamma11$), nidogen (entactin/nidogen), and heparan sulfate (primarily perlecan and agrin). It is a layer of acellular basement membrane that is 300–350 nm in thickness and plays a role in glomerular filtration as well as podocyte adhesion, migration, and differentiation. Such a molecular structure endows the GBM with the dual functions of being the mechanical barrier and charge barrier, and abnormalities in each role may lead to proteinuria.

4.1. Collagen Type IV and Proteinuria. Collagen type IV is a triple helix protein composed of three α chains. Its molecular weight is 180 kDa, and it consists of isomeric chains ($\alpha1$–$\alpha6$) encoded by six different genes. These genes form the reticular structure via intermolecular interactions, shaping the fundamental skeleton of the GBM; other molecules attach to it in different ways. During human fetation, collagen type IV is dominated by $\alpha1.\alpha1.\alpha2$ tripolymer-originating meshwork in the earliest stage of forming the GBM vascular loop; however, with the gradual development and maturity of the glomerular capillary loop, collagen type IV is gradually replaced by $\alpha3.\alpha4.\alpha5$ tripolymer-originating meshwork. The alteration in collagen type IV during fetation is thought to be related to oxidative and physical stress. In the kidneys, since plasma proteins contain a variety of proteases that contact the GBM directly and the $\alpha3.\alpha4.\alpha5$ tripolymer is rich in disulfide bonds, the $\alpha1.\alpha1.\alpha2$ tripolymer may be more resistant to the effects of proteases and various physical stimuli. When gene mutations occur in the $\alpha1$-$\alpha5$ chain, the GBM develops irregular pachynsis, multiple stratifications, and a reticular structure. This is manifested as hereditary nephropathy-Alport syndrome, which is clinically

characterized by progressive hematuria, proteinuria, and renal failure. If the gene encoding $\alpha 4$ chain is mutated, "thin basement membrane disease" can occur, which is characterized by hematuria under the microscope and is also known as "benign familial hematuria." When autologous antibodies are present in the NCI structural region of the anticollagen type IV $\alpha 3$ chain, the GBM mechanical barrier is disrupted and produces massive proteinuria. This is clinically referred to as "Goodpasture syndrome" [14–16].

4.2. Laminin and Proteinuria. Laminin, secondary only to collagen in GBM content, is a heterotrimeric glycosidoprotein composed of α, β, and γ chains. It is cross-shaped and provides the supporting structure for other parts of the GBM. It is believed that there are five α chains, three β chains and two γ chains in humans, forming 11 different types of laminin tripolymer. Mutations in the LAMB2 gene, encoding the laminin $\beta 2$ chain, are associated with the Pierson's syndrome. The human LAMB2 gene maps to chromosome band 3p21 and is composed of 32 densely packed exons spanning about 12 kb of genomic DNA [17]. Mutations in the LAMB2 were also found in patients with congenital nephrotic syndrome [18], and LAMB2 mutations were reported to result in loss of laminin-$\beta 2$ expression in the kidney [19]. LAMB2 knockout mice, which model is Pierson syndrome, show congenital albuminuria followed by podocyte foot process effacement, and they die at about 3 weeks of age with severe neuromuscular defects and nephrotic syndrome [20]. A recent case reported a minor variant of Pierson syndrome in a teenage girl with severe myopia since early infancy and proteinuria first detected at age 6. At the age of 11 she was found to carry a unique homozygous nontruncating LAMB2 mutation [21].

Similar to collagen type IV, during the development of the kidney, a series of changes occur to the upper laminin layer of the GBM, from laminin-10 ($\alpha 5\beta 1\gamma 1$) in the fetal period to laminin-11 ($\alpha 5\beta 2\gamma 1$) in the mature period. Laminin-11, which contains $\beta 2$, is a glycosidoprotein that is indispensable for maintaining the function of the GBM. Although $\beta 2$ knockout mice have an intact GBM ultrastructure, when GBM functions are severely damaged, the mice develop massive proteinuria seven days following birth and extensive pathologically fusion of glomerular podocytic processes occur, similar to the minute lesions in human beings. In addition, the emergence of proteinuria occurs in advance of subpodocytic fusion and SD disappearance, and the mice die 3–5 weeks after birth. This indicates that laminin may play an important role in cell-matrix interaction [22]. Laminin interacts with the various components of the GBM such as agrin, nidogen, and perlecan and is associated with cell surface receptors such as integrin $\alpha 3\beta 1$, $\alpha 6\beta 1$ and α-dystrophin, which constitutes the molecular biological basis for laminin's participation in cell-matrix interactions [22].

4.3. Nidogen and Proteinuria. Nidogen, also known as entactin, is a single-stranded glycosidoprotein of 150 kDa in molecular weight. It was originally extracted from the extracellular matrix of an Engelbreth-Holm-Swarm (EHS) tumor. Nidogen consists of three spherical regions (G1, G2, and G3), and region G3 associates noncovalently with the $\gamma 1$ chain of laminin in an equimolar fashion. Region G2 associates with collagen type IV in a manner similar to how the collagen stroma is linked with the laminin meshwork. This region also binds to basement membrane proteoglycans. Therefore, through its ability to crosslink other GBM components, nidogen plays an important role in the development and maintenance of GBM integrity.

4.4. Heparan Sulfate and Proteinuria. Proteoglycans on the GBM are dominated by heparan sulfate (HS) polysaccharide. This includes proteoglycans such as perlecan and agrin, which are distributed in the GBM. Heparan sulfate is rich in negative charges that can limit the ability of negatively charged plasma proteins to pass. It is therefore an important participant in the glomerular charge barrier. HS may also interact with collagen and laminin in the GBM to maintain the structural integrity of GBM molecules. It may therefore also exert an effect as a mechanical barrier [23]. It has been found in animal experiments that one-off intravenous injection of HS monoclonal antibodies or digestion of HS with heparinase in rats may both induce massive proteinuria in rats. Data from perlecan N-terminal gene-knockout mice has shown that there are no obvious morphological changes to the kidneys of these mice under physiological conditions, but their kidneys are highly sensitive to protein load. When injecting bovine serum albumin into these mice, massive proteinuria emerges [23]. In addition, studies on agrin show that it may promote the anions binding to the GBM. These studies show that perlecan and agrin play important roles in maintaining the GBM charge barrier and mechanical barrier.

5. Podocytes and Proteinuria

In podocytes and podocytic processes, SDs are important components of the glomerular filtration barrier. Slit diaphragms are zipper-like membranaceous electrodense structures that are linked in a zigzag fashion among podocytic processes. Most studies show that SDs have a rigid structure with a relatively constant diameter (20–50 nm) and are composed of multiple protein complexes. The bridging adjacent podocytic processes are the last barriers of glomerular filtration. The slit membrane can be divided into three parts: an extracellular part (the extracellular portion of the transmembrane protein on the slit membrane), a transmembrane part (the intramembrane part of the transmembrane protein and the membrane-associated protein on the podocytic membrane), and an intracellular part (the cytoplasmic domain of the transmembrane protein, and other membrane-related proteins and slit membrane proteins in podocyte). A number of SD molecules expressed by podocytes have recently been discovered and can be classified into four categories: SD membrane proteins including Nephrin, podocyte skelemins including α-actinin-4, basement membrane-podocyte junctional membrane proteins including $\alpha 3\beta 1$ integrin and podocyte terminal membrane proteins including Podocalyxin [24]. In depth study of the structural molecules of

podocytes may accelerate further understanding of the structure and function of the glomerular filtration barrier and may clarify the pathogenesis of proteinuria.

5.1. Nephrin and Proteinuria. Nephrin was the first SD molecule found in podocytes. It is the transmembrane protein encoded by the NPHS1 gene and is composed of 1241 amino acids. It belongs to the immunoglobulin superfamily of cell adhesion molecules and is located specifically in the glomerular slit diaphragm region. The extracellular amino terminal region of nephrin molecules has eight Ig repetitive sequences, one interval region and one type III fibronectin-like region. Each Ig motif contains two cysteines (Cys), which may form a disulfide bond in the Ig repetitive structure, endowing the Ig motif with a spherical or elliptical form. If the Ig motif forms a chain-like structure, the slit diaphragm is the widest part of the structure (35–45 nm). In addition to the two Cys residues contained in each Ig motif, the nephrin molecule also has three free Cys: one located in the first Ig motif, one in the interval region and one in the fibronectin-like region. A Cys in the first Ig motif forms a disulfide bond with another Cys in the interval region of the nephrin molecule; the two molecules show homophilic adhesion. In this way, many nephrin molecules form axial filaments along the slit diaphragm. In addition, a Cys in the fibronectin-like region may form a disulfide bond with another nephrin molecule or with an unknown protein connecting the slit diaphragm and the cytoskeleton. In summary, the three free Cys residues participate in the formation of intermolecular disulfide bonds that enhance the integrity of the slit diaphragm. Deletion of these three Cys residues may relax the slit diaphragm, leading to its disappearance, disrupting the filtration barrier and inducing proteinuria [25].

A gene mutation in nephrin can lead to congenital nephrotic syndrome of the Finnish type (CNF), with clinical features including massive intrauterine proteinuria, a placental weight exceeding the body weight by 25% at birth and significant edema. This syndrome is progressive, and patients usually die within 2 years after birth. At the first day after intravenous injection of extracellular antinephrin monoclonal antibody (mAb)5-1-6 into rats, massive proteinuria has been shown to emerge and to peak on day five. Studies on models of nephrotoxic serum nephritis, Heymann nephritis, and amino nucleotide nephropathy determined that the expression of nephrin in renal structures of mice was significantly decreased in the model group. Moreover, the mice developed massive proteinuria [25, 26].

5.2. Podocin and Proteinuria. Podocin is an integral membrane protein that was detected while studying steroid-resistant congenital nephrotic syndrome using targeted cloning technology. It is encoded by the NPHS2 gene [27]. Podocin is a new member of the stomatin family of lipid raft-associated proteins and consists of 383 amino acids with the molecular weight of 42 kDa and a "hairpin-like" single membrane region. Its N- and C-termini are located in the cytoplasm. Podocin interacts with nephrin and CD2AP via its C-terminus. It plays an important role in maintaining the structure and function of SDs as a supporting protein. Studies in zebra fish found that at 72 and 96 h after fertilization, the expression of glomerular podocin protein decreased or disappeared, leading to abnormalities in slit diaphragms and preventing the formation of normal podocytic processes [27].

NPHS2 gene-encoding podocin knockout mice showed proteinuria prior to birth and died within several days following birth; their pathological manifestation was severe mesentery sclerosis. Extensive subpodocytic fusion and slit membrane disappearance could be observed via electron microscopy. NPHS2 gene mutations have been detected in syndromes such as human congenital familial steroid-resistant nephrotic syndrome and sporadic steroid-resistant nephrotic syndrome [28–30], suggesting that podocin plays an important role in maintaining the structure of podocytic processes and the integrity of slit membranes. Podocin could be of great importance in the pathogenesis of proteinuria.

5.3. CD2AP and Proteinuria. CD2AP is a transmembrane protein belonging to the immunoglobulin superfamily and is composed of 639 amino acids with a molecular weight of 80 kDa. It was initially determined that, as an intracellular ligand of T cell and natural killer cell CD2 receptors, CD2AP can stabilize connections between T cells and antigen-presenting cells. Furthermore, CD2AP is extensively expressed in various structures of humans and mice, and it has been shown through immunoelectron microscopy that CD2AP localizes near the intracellular segment of nephrin in the podocyte side wall. Simultaneous immunofluorescence confirmed that CD2AP is primarily expressed in the glomerulus of the kidney and located at the podocyte slit membrane within the glomerulus and can also be detected in the concentrated pipe as well as some proximal and distal tubules. Its N-terminus contains a Src homology 3 (SH3) domain that can identify the proline-rich amino acid sequence in the intracellular C-terminus of CD2. The middle segment is a proline-rich structural domain that has a variety of loci and can bind with a number of enzymes and protein molecules. The C-terminus has the binding sites for the helical structure region and the actin cytoskeleton; the leucine zipper domain in the C-terminus can regulate the development of the CD2AP homodimer [31, 32]. As an adaptin, CD2AP can interact with slit membrane proteins such as podocin and nephrin via its C-terminus, locating its anchor within subpodocytic lipid rafts in order to preserve the functions of the cytoskeleton and SD. Damage to CD2AP not only affects the function of SDs, but also directly damages the podocyte cytoskeleton, disrupting the stability of the cytoskeleton and leading to pathological changes such as subpodocytic deformation and disappearance, thus inducing massive proteinuria [33]. In addition, CD2AP can interact with various signaling molecules via its SH3 region and participate in cytoskeletal assembly. The CD2AP gene-knockout mouse develops subpodocytic fusion or disappearance one week after birth, mesangial cell proliferation, extracellular matrix deposition and proteinuria at week two, and nephrotic syndrome at weeks three or four and dies of proteinuria and renal failure as a result of subpodocytic

defects at weeks six or seven. This indicates that CD2AP is of great importance for maintaining the structure of podocytes and SDs [31, 32]. Nevertheless, no nephrin abnormalities were detected in this model at the genetic level or with respect to protein localization, suggesting that the interaction between CD2AP and nephrin is not necessary for the generation and aggregation of nephrin. Only when severe glomerular injury occurs can changes be observed in nephrin, which might be related to the disappearance of the subpodocytic slit diaphragm. Another study showed that nephrin was expressed earlier than CD2AP and that CD2AP gene-knockout mice showed a gradual disappearance of podocytic processes that were previously normal in appearance. These findings suggest that nephrin can induce podocytic processes independent of CD2AP, but CD2AP is of great significance for maintenance of the morphology and function of the subpodocytic slit diaphragm [26].

5.4. NEPH1 and Proteinuria. NEPH1 is a transmembrane protein with a molecular weight of 110 kDa. The human Neph1 gene is located in chromosome 1 (1g21-q25), within a known gene domain relating to childhood nephrotic syn-drome. The NEPH family contains three members: NEPH1, NEPH2, and NEPH3. They all belong to the immunoglobulin superfamily and have five similar extracellular immunoglobulin-like repetitive sequences, a transmembrane region and an intracellular region composed of 198–235 amino acids. NEPH1 is extensively distributed in many tissues of humans and mice; its expression level is highest in the kidney. Immunohistochemical staining has shown that the molecule is only expressed in podocytes and immunoelectron microscopy confirmed that the molecule is localized to the slit membrane [34, 35]. Neph1 knockout mice demonstrated similar phenotypes to nephrin knockout mice. At 1 week after birth, they were weaker and smaller than normal mice and without edema; however, with the development of the disease, almost all of the mice developed proteinuria to varying degrees and died 3-4 weeks after birth. Electron microscopy showed the existence of subpodocytic fusion, suggesting a significant role for Neph1 in maintaining the integrity of the glomerular filtration barrier [36].

Nephrin and NEPH1 gene deletions can lead to similar phenomena, such as subpodocytic fusion, proteinuria, and high perinatal mortality, suggesting that they may have the same pathological mechanism. Immunocoprecipitation showed that the extracellular segment of nephrin can interact convergently and divergently with the extracellular region of NEPH1 and its own extracellular region, respectively, but that NEPH1 does not interact with its own extracellular region. This indicates that nephrin and NEPH1 form a kind of heterogeneous oligomer receptor complex on the subpodocytic slit diaphragm by cis-trans interactions which participates in the formation of the SD zipper-like structure and maintains the normal structure of podocytes and the integrity of SDs. This interaction is rather complex and involves multiple immunoglobulin-like structural domains of the two molecules [35, 37].

Podocin has a tissue distribution similar to that of the NEPH family. Immunocoprecipitation showed that the intracellular regions of the three members of the NEPH family can all bind to the podocin C-terminus. Podocin can also precipitate endogenous NEPH1 originating from podocytes. The intracellular segments of proteins in the NEPH family all have a highly conserved sequence composed of nine amino acids (KDPTNGYYxV). NEPH1 gene mutation or replacement of the no.7 tyrosine of the conserved sequence with an alanine can block the association of NEPH1 and podocin, confirming that the integrity of this conserved segment is of great importance for the interaction between podocin and NEPH1. The specific mechanism of podocin and Neph1 interaction involves dephosphorylation of tyrosine 637 of NEPH1 via Tec kinase, which promotes the association of NEPHI and the podocin carbon terminus. The interaction between NEPH1 and podocin plays a significant role in the maintenance of podocyte SDs [38].

5.5. α-Actinin-4 and Proteinuria. Four members of the α-actinin family have thus far been identified: α-actinin-1 (nonmuscle type), α-actinin-2 (muscle type), α-actinin-3 (muscle type), and α-actinin-4 (nonmuscle type). Only α-actinin-4 expression has been found in renal tissues, primarily in podocytes, and its coding gene, ACTN4, is located on chromosome 19q13. α-actinin-4 is an actin filament cross-linked protein with a molecular weight of approximately 100 kDa, and it is an antiparallel homodimer and is dumbbell-shaped (width: 4-5 nm, length: 40–50 nm). It consists of three structural domains: the N-terminal CH domain is an actin binding domain (ABD) containing 250 amino acid residues. The C-terminal 150 amino acid residues constitute the CaM domain, containing two "EF hand" repeats. The central α-helix repetitive fragment is comprised of the four spectrin repeats from R1-R4, each fragment consisting of 122 amino acid residues forming a rod-like structural domain. Podocyte α-actinin-4 is formed through interaction between the two identical peptide chains via the central spectrins. α-actinin-4 can regulate actin polymerization and depolymerization. It bundles the loose actin fibers in podocytes into fasciculi with contraction through the ABD structural domain on both ends in order to stabilize podocyte cytoskeletal structures such as actin and actin filaments, maintain the morphology of podocytic processes, and regulate the movement of the cytoskeleton [39, 40]. α-actinin-4 is extensively expressed in podocytes. In experimental nephrotic syndrome as well as primary and secondary human glomerular lesions, α-actinin-4 is expressed and abnormally localized, along with abnormal expression of other SD-related proteins [41]. Gene mutations in ACTN4 may lead to focal segmental glomerulosclerosis, suggesting that the actin cytoskeleton may affect the structure or function of podocytes and participate in the occurrence and development of proteinuria [42]. α-actinin-4 deletion in mice showed progressive proteinuria, and these mice died within a few months of birth. Early electron microscopy results showed regional subpodocytic fusion which later became diffuse fusion, and indicated that the structure of the SDs was severely disrupted [43]. These results indicate that α-actinin-4 is of crucial importance for the maintenance of cytoskeleton and SD functions.

5.6. α3β1 Integrin and Proteinuria. Integrins are a class of molecules belonging to the transmembrane glycosidoprotein cell surface receptor family and are heterodimers formed by α and β subunits through noncovalent bonds. To date, 16 α subunits and nine β subunits have been identified, which together assemble into at least 19 kinds of integrins. According to differences in β subunits, the integrin family can be divided into three subfamilies: β1, β2, and β3. The kidneys are dominated by the β1 subfamily, including α3β1 integrin and others. In kidneys, α3β1 integrin is highly expressed in podocytic processes along the glomerular basement membrane, and the molecules exist extracellularly in the form of membrane proteins and contain a transmembrane region and an intracellular region. The α3 subunit has the molecular weight of 150 kDa and is encoded by the ITGA3 gene located on chromosome 17. It has been found that this subunit contains a site for binding calcium ions. The β1 subunit has four Cys-rich repeats whose macrocycles at the terminal extracellular amino acids are reinforced by intrachain disulfide bonds (S-S). There is also an integrin-linked kinase (ILK) on this subunit. When podocytes are injured, the activated ILK phosphorylates the intracellular region of the β1 subunit, thereby reducing the binding between α3β1 integrin and the basement membrane. The globular regions at the terminal amino acids of the integrin α3 and β1 subunits interact with each other, forming the extracellular ligand-binding site, and bind with the basement membrane laminin, collagen IV and fibronectin at focal contacts. The cytoplasmic domains of the two subunits, however, are comparatively shorter and bind with subpodocytic cytoskeletal secondary filaments mediated by actin auxiliary protein molecules such as Talin, Vinculin, and Paxillin. The formed "basement membrane-integrin-cytoskeleton" structure not only stabilizes the podocyte cytoskeleton, but also initiates the integrin-dependent signaling pathway as a transmembrane information system. This affects cell morphology and the cell cycle, regulates gene expression and cytoskeletal assembly and contraction, and modulates cell proliferation, differentiation, and apoptosis [44, 45]. Animal experiments showed that anti-α3β1 integrin antibodies can separate podocytic processes from the GBM and induce proteinuria. In human FSGS patients and in puromycin-aminonucleoside- (PAN-) induced nephropathy mouse models, there were significantly downregulation of α3β1 integrin expression than in the normal control group. Moreover, their expressions were clearly decreased prior to the morphological changes in the podocytes. It is therefore clear that α3β1 plays an important role in maintaining the normal morphology and functioning of podocytes [46].

5.7. TRPC6 and Proteinuria. Transient Receptor Potential Cation channel 6 (TRPC6) is a hexametric transmembrane protein with intracellular N- and C-termini and a pentameric and hexametic transmembrane structure constituting a nonselective cation channel. The TRPC family can be divided into four subgroups according to structural homology and functional specificity: TRPC1, TRPC2, TRPC4/5, and TRPC3/6/7. A large number of TRPC6 proteins are distributed in brain tissues, with some in the lung and ovary. In the vascular system, TRPC6 is distributed in smooth muscle cells and endothelial cells and participates in the regulation of vascular smooth muscle function. TRPC6 is expressed in the glomerulus and renal tubules, but is primarily localized in podocytes. Immunogold labeling has shown that TRPC6 is located in primary and secondary podocytic processes, especially around the SD annex. Immunofluorescence double labeling showed that podocyte TRPC6 is colocalized with Nephrin, Podocin, and CD2AP, and immunoprecipitation showed that TRPC6 interacted with Nephrin and Podocin, but not with CD2AP [47, 48].

TRPC6 knockout mice primarily exhibited an elevation in blood pressure and increased arterial ring contraction induced by the agonist, indicating that TRPC6 plays a rather important role in regulating vascular smooth muscle function [49]. A TRPC6 gene mutation may cause familial FSGS, as the mutation detection rate of TRPC6 was 7% in familial FSGS. A major clinical manifestation of proteinuria has also been observed. The majority of patients develop end-stage renal diseases about 10 years after onset, and pathological renal manifestations are common in FSGS. Studies on secondary FSGS found that TRPC6-originating calcium influx leads to the abnormal localization of Nephrin in the SD, such that Nephrin is unable to function normally, leading to changes in TRPC6-mediated calcium currents, which are critical in the regulation of intracellular molecules and cytoskeletal behavior in podocytes [49, 50].

5.8. Megalin/gp330 and Proteinuria. Megalin/glycosidoprotein (gp330) is a receptor involved in multiple-ligand-mediated endocytosis. It is located on one side of podocytes, as well as in microvilli of clathrin-coated fovea and proximal convoluted tubules. It belongs to the LDL receptor family, and its ligands include apoE-rich β-VLDL, lipoprotein (a), lactoferrin, oprotein lipase, aprotinin, plasminogen and others. Under normal conditions, megalin on podocytes can bind with proteins filtered from the GBM and degrade them via endocytosis. Heymann nephritis is an experimental model of human membranous nephropathy. In this model, the following reactions are triggered after binding occurs between a megalin antibody and megalin: (1) an antigen antibody complex-activating alexin cascade, resulting in the formation of C5b-9 membrane attack complex and exerting cytolytic toxicity; (2) enhanced expression of subsolvent NADPH oxidases in podocytes, which are activated and translocated to the cell membrane, generating a large number of reactive oxygen species; and (3) apoE and apoB100 aggregation that is observed at sites of regional aggregation and regional immune complex deposition of various megalin ligands, which are all megalin ligands. apoE and apoB100 undergo peroxidizing modifications as a result of the effects of reactive oxygen species produced during the cytotoxic process initiated by alexin. Lipid peroxidation leads to glomerular capillary wall injury, causing proteinuria that is clear alleviated after treatment with probucol, an inhibitor of lipid peroxidation [51].

5.9. WT1 and Proteinuria. Another well-described genetic defect in patients with primary nephrotic syndrome is

the spectrum of clinical pictures caused by mutations in Wilms tumor suppressor gene 1 (WT1), a transcription factor regulating the expression of many genes through DNA binding [52]. WT1 was identified by positional cloning in children with the WAGR syndrome, a syndrome characterized by the association of Wilms' tumor (W), aniridia (A), genitourinary malformations (G), and mental retardation (R) [53]. The WT1 gene contains 10 exons and spans approximately 50 kb on chromosome 11. It generates a 3 kb mRNA and encodes a 52–54 kDa protein [54]. In addition to being a tumor suppressor gene, WT1 has been shown to play crucial roles during embryogenesis, especially during kidney development [55]. WT1 mutant mice do not form kidneys and mice lacking the transcriptionally active WT1 splice variant WT1-KTS develop kidneys with very few immature glomeruli [56]. The WT1 gene is widely expressed in epithelial cells of early nephron and is restricted to podocytes in the mature glomeruli [57]. Based on this, WT1 is often utilized as a molecular marker for evaluating podocyte number and density under different circumstances [58]. Several lines of evidence suggest that WT1 may indeed play an important role in the maintenance of normal podocyte function [55]. Heterozygous de novo mutations in WT1 cause Denys-Drash syndrome (DDS) and Frasier syndrome (FS) [59]. WT1 is mutated in 94% of all Denys-Drash syndrome (DDS) patients, companied with the development of glomerular nephropathy involving glomerulosclerosis [55]. WT1 mutations have also been found in patients with nephrotic syndrome and isolated cases of glomerulosclerosis [57, 60]. In addition, WT1 is downregulated in a variety of glomerular diseases with podocyte injury, and WT1 mRNA is detected in the urine of some patients with glomerular diseases [61]. WT1 plays a fundamental role in controlling the expression of major podocyte-specific genes such as nephrin and podocalyxin in adult kidney [62, 63]. Although it has been implicated that changes in the expression of TGF-β1, PDGF-α, and Pax-2 which are regulated by WT1 affect cytoskeletal architecture [64], the complete set of WT1's targets in podocytes remains to be defined.

5.10. PLCE1 and Proteinuria. PLCE1 (phospholipase C epsilon-1) gene locates at chromosome 10q23.32-q24.1, and its encoded protein-phospholipase Cε1 (PLCε1) is a member of phospholipase C (PLC) family [65]. PLCε1 is a phospholipase enzyme that catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate and generates two second messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), which then initiate a cascade of intracellular responses that result in differential gene expression, cell growth, and differentiation [58]. PLCε1 expresses in the matured podocyte of renal glomerulus and plays an essential role in the formation and normal development of capillary loop of glomerulus [65]. The role of PLCE1 in renal pathophysiology remains complicated. Mutations in PLCE1, which was identified as a new cause of autosomal recessive nephritic syndrome in children that present with diffuse mesangial sclerosis (DMS) and FSGS, cause arrest of glomerular podocyte development at the S-shaped stage, thereby halting glomerular development and causing nephrotic syndrome [65, 66]. But enhanced signalling through a form of PLC within podocytes results in podocyte injury and proteinuria [67]. It has been shown that PLCε1 interacted with H-Ras, IQGAP1 (IQ motif-containing GTPase-activating protein 1) and BRAF (v-raf murine sarcoma viral oncogene homolog B1), then serving as crucial intermediates in many signaling pathways [68]. Identification of additional proteins that are expressed in the podocyte and interact directly or indirectly with PLCε1 will be needed to help in the understanding of how mutations in PLCE1 cause nephrotic syndrome.

5.11. Lmx1b and Proteinuria. Lmx1b is one of a family of more than nine LIM-homeodomain genes regulating gene transcription via its interactions with gene promoter and enhancer sequences, in conjunction with other transcription factors [69]. Mutations in Lmx1b cause nail-patella syndrome (NPS), an autosomal dominant disease with skeletal abnormalities, nail hypoplasia, and nephropathy [70]. Renal involvement occurs in 25% to 60% of cases, ranging from nonnephrotic proteinuria to end-stage renal disease [71]. Ultrastructurally, foot process effacement was observed for a certain percentage of podocytes [72]. It has been reported that Lmx1b is required for normal podocyte differentiation [73]. Lmx1b $-/-$ mice exhibit kidney defects as well as patterning defects in appendicular skeletal structures and associated soft tissues, and they die shortly after birth [74]. It has been demonstrated that the transcription of podocin is mainly regulated by the transcription factor Lmx1b, which binds to a FLAT-F element and displays enhancer function [75]. However, a study of a podocyte-specific Lmx1b knockout showed later development of proteinuria and greater expression of type IV collagen chains and podocin [76].

5.12. SMARCAL and Proteinuria. Mutations in SMARCAL1 (SWI/SNF-related, matrix-associated, actindependent regulator of chromatin, subfamily a-like 1) are involved in the development of Schimke immunoosseous dysplasia (SIOD). This autosomal recessive disorder is characterized by the autosomal recessive transmission of spondyloepiphyseal dysplasia and characteristic dysmorphic features, lymphocytopenia and/or T-cell immunodeficiency, and renal dysfunction including proteinuria and nephrotic syndrome due to FSGS [77]. Two families have been reported in which siblings of affected individuals have incomplete penetrance of SIOD [78, 79]. Furthermore, mutations in SMARCAL1 were also found in two siblings with an incomplete phenotype of SIOD. The siblings were initially classified as suffering from familial steroid-resistant nephrotic syndrome [80]. As SMARCAL1 encodes a SWI/SF2-related protein involved in chromatin remodeling [81], it is tempting to speculate that SMARCAL1 regulates expression of podocyte proteins. However, podocyte genes potentially regulated by SMARCAL remain to be identified.

5.13. MYH9 and Proteinuria. An exceptional example of the genetic complexity of nephrotic syndrome was shown by two independent studies demonstrating a strong association of common genetic variants in the MYH9 gene with FSGS and hypertensive ESKD [82, 83]. MYH9 encodes for

the heavy chain of nonmuscle myosinIIA (NMMHC-IIA). MYH9 is abundantly expressed in glomeruli, and mainly in podocytes [84]. It has been reported that NMMHC-A acts as a component of the podocyte cytoskeleton, contributing to its contractile functions [85]. A growing body of evidence indicates that loss of MYH9 function may be sufficient to cause kidney disease. Podocyte-specific deletion of MYH9 mice is predisposed to Adriamycin-induced glomerular injury, including podocyte effacement, glomerulosclerosis and proteinuria [86]. Recently, it has been demonstrated that podocytes host response to HIV-1 includes downregulation of MYH9 expression, and this downregulation might play a role in the pathogenesis of HIVAN [87]. Furthermore, MYH9 polymorphisms are associated with diabetic nephropathy in European Americans [88]. However, the underlying pathophysiologic events occurring at the chronic kidney disease associated with MYH9 high-risk haplotypes remain unknown.

5.14. SCARB2 and Proteinuria. Mutations in the lysosomal membrane protein human scavenger receptor class B, member 2 (SCARB2), have recently been found to cause action myoclonus renal failure syndrome (AMRF) in humans, which is characterized by collapsing FSGS and progressive myoclonic epilepsy [89]. Clinical report of two siblings revealed that AMRF resulted from a mutation in the SCARB2 gene and the renal involvement was due to nephropathy C1q [90]. SCARB2 (Limp-2 in mice) encodes for the ubiquitously expressed lysosomal integral membrane protein type 2 (LIMP-2) mainly found in lysosomes and late endosomes [91]. This LIMP-2 protein has been shown to act as a receptor to bind β-glucocerebrosidase which is a lysosomal enzyme deficient in most cases of Gaucher disease [92]. LIMP-2 knockout mice have tubular proteinuria due to an inability to fuse lysosomes with endosomes and degrade reabsorbed proteins [93]. Recently, although novel SCARB2 mutation has been found in AMRF [94], the pathophysiologic events leading to glomerular disease in cases with SCARB2 mutations remain unknown.

5.15. Other Podocyte-Related Molecules and Proteinuria. The components of SDs also include many other podocyte-related molecules, such as Densin, which binds to the cytoskeleton to maintain the polarity of podocytes and interacts with podocalyxin, megalin, and α-actinin-4 within podocytes. Moreover, Densin participates in the pathogenesis of proteinuria. Glomerular epithelial cell protein 1 (GLEPP-1) is a newly discovered receptor-like membrane protein tyrosine phosphatase (RPTP). In the kidney, it is specifically expressed at the plasma membrane on top of podocytic processes. P-cadherin plays a role in connecting SD structural molecules. Galloway Mowat Syndrome (GMS) is a rare autosomal recessive disorder comprising of nephrotic syndrome with central nervous system involvement [95]. Linkage studies in two Algerian families identified a homozygous mutation in the GMS1 gene [52]. Recent exome sequencing as well as a whole-genome linkage analysis revealed MYO1E mutations in childhood proteinuric disease and FSGS [96]. MYO1E appears to be important for podocyte motility and may also stabilize the podocyte cytoskeleton [97]. In addition, synaptopodin is an actin-associated protein essential for the integrity of the podocyte actin cytoskeleton because synaptopodin-deficient mice display impaired recovery from protamine sulfate-induced foot process effacement and lipopolysaccharide-induced nephrotic syndrome [98]. Besides, recent studies found that the normal expression of CD38 importantly contributes to the differentiation and function of podocytes and the defect of this gene expression may be a critical mechanism inducing EMT and consequently resulting in glomerular injury and sclerosis [99], and CD151 as a crucial modifier of integrin-mediated adhesion of podocytes to the GBM [100]. However, the functions of these molecules remain undefined and require further study.

6. Mitochondrial Disorders and Proteinuria

Mitochondrial DNA plays a crucial role in oxidative production of energy. Thus, defects in mitochondrial DNA can affect virtually all organ systems. Mitochondrial DNA mutations have been recently described also in association with kidney disease, mainly focal and segmental glomerulosclerosis [101].

6.1. CoQ10 and Proteinuria. Coenzyme Q10 (CoQ10) is a lipophilic molecule that transfers electrons from mitochondrial respiratory chain complexes I and II to complex III [102]. CoQ10 deficiency is associated with a variety of clinical phenotypes, including nephrotic syndrome [103]. COQ2 mutations have been identified in patients presenting with early-onset NS, severe oliguric renal failure and collapsing glomerulopathy [104]. Dietary supplementation with Q10 provides a dramatic rescue of both proteinuria and interstitial nephritis in the interstitial nephritis mice model [105]. Moreover, administration of CoQ10 had significant beneficial effects on albuminuria in the experimental model of type 2 diabetes, db/db mice [106]. Recently, CoQ10 prevented altered mitochondrial function and morphology, glomerular hyperfiltration and proteinuria in db/db mice, highlighting the role of mitochondria in the pathogenesis of diabetic nephropathy and the benefits of preventing increased oxidative stress [107].

6.2. tRNA Mutation and Proteinuria. The point mutations in the transfer tRNA Leu(UUR) gene are mainly associated with the mitochondrial encephalomyopathy, lactacidosis and stroke-like episode (MELAS) syndrome [108]. An A to G transition at nucleotide position 3243 in the mitochondrial tRNA Leu(UUR) gene has showed a pathogenic effect in maternally inherited diabetes and deafness and progressive kidney disease [109]. A3243G mutation is also found in patients with FSGS sometimes associated with maternally inherited diabetes and/or sensorineural hearing loss [110]. In a report of a boy with A3243G mutation in the tRNA Leu (UUR) gene, proteinuria was detected at the age of 6 years, including large amounts of low-molecular-weight proteins such as beta(2)- and alpha1-microglobulin [111].

A3243G mutation was also found in the case of a 59-year-old male with a personal and maternal history of diabetes and deafness, who presented with cardiomyopathy and kidney disease [112]. Furthermore, other mutations in mitochondrial tRNA genes were described in patients with mitochondrial cytopathy presenting with FSGS [113, 114].

7. Circulating Factors and Proteinuria

7.1. Angiotensin II and Proteinuria. Angiotensin II, traditionally playing a central role as a mediator of glomerular hemodynamic adaptation and injury, is now recognized to exert proinflammatory action leading to upregulation of chemokines, adhesion molecules, and other fibrogenic growth factors [115]. Podocytes are a direct target for angiotensin II—mediated injury by altered expression and distribution of podocyte proteins. Rats receiving angiotensin II by minipump developed hypertension in association with proteinuria. Both real-time PCR and quantitative in situ hybridization demonstrated a significant increase in nephrin gene expression in angiotensin II infused animals compared with control animals [116]. Angiotensin II promotes podocyte injury indirectly by increasing calcium influx and production of reactive oxygen species [117]. Angiotensin II is also closely related to vascular endothelial cells. Under physiological conditions, it regulates the development, maturation, and permeability of endothelial cells [118]. Angiotensin I and angiotensin II, both exert their effects via the Tie2 receptor on endothelial cells. The function of angiotensin I lies in stabilizing endothelial cells and preventing inflammatory responses, angiogenesis, and endothelial cell permeability from increasing. On the other hand, angiotensin II primarily exerts its antiangiotensin I effect via binding to the Tie2 receptor.

7.2. VEGF and Proteinuria. Vascular endothelial growth factor (VEGF) is a 43–46 kDa glycoprotein that serves as a key survival factor for vascular endothelium [119]. Through binding with the VEGFR on endothelial cells, VEGF regulates angiogenesis and endothelial cell permeability. In kidneys, angiotensin I and VEGFs, secreted by podocytes, bind to the glomerular vascular endothelial cell receptor Tie2 and VEGFR, affecting the phenotype of endothelial cells and the function of their filtration barrier. Circulating physiological levels VEGF is important for the homeostasis of kidney glomerulus. Blocking VEGF signal transduction by anti-VEGF antibody or soluble receptors could lead to proteinuria. An increase in the incidence of proteinuria has been found in patients receiving anti-VEGF antibody treatment. Moreover, neutralizing VEGFs during blood circulation in mice treated with equimolar anti-VEGF antibody or VEGF receptor may induce proteinuria. At this time point, the major lesion is located in endothelial cells, manifested as vacuolar degeneration of endothelial cells and detachment from GBM. These results suggest that VEGFs may play a significant role in the pathogenesis of proteinuria [120].

7.3. Other Circulating Factors and Proteinuria. Fibroblast growth factor 21 (FGF21) is a hepatic hormone involved in the regulation of lipid and carbohydrate metabolism. Plasma FGF21 levels are significantly increased with the development of early- to end-stage CKD and are independently associated with renal function and adverse lipid profiles in Chinese population [121]. Furthermore, it has been reported that serum soluble urokinase receptor (suPAR), which is elevated in two-thirds of subjects with primary FSGS, may cause FSGS [122]. A recent study investigated in 48 stage-2-to-4 CKD patients showed that circulating endogenous inhibitor of NO synthase asymmetric dimethylarginine (ADMA) emerged as an independent correlate of proteinuria [123]. Previous study from our group found the mineralocorticoid aldosterone infusion in mice could induce urinary protein excretion and podocyte injury [124]. Although increasing evidence determined that circulating factors played a role in proteinuria, the precise nature of these factors and the mechanisms by which they cause renal injury need further studies.

8. Signaling Pathways and Proteinuria

8.1. mTOR Signaling and Proteinuria. Mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase, which controls cell growth and metabolism in response to nutrients, growth factors, cellular energy, and stress. mTOR inhibitors rapamycin clinically used for immunosuppression after organ transplantation can cause renal deterioration and proteinuria after conversion from calcineurin inhibitors [125]. mTOR is a widely expressed protein that mediates its functions in two complexes, mTOR complex 1 (mTORC1) and mTORC2 [126]. Podocyte- specific mTORC1 activation in diabetic mice recapitulated many diabetic nephropathy features, including podocyte loss, GBM thickening, mesangial expansion, and proteinuria in addition to podocyte loss [127]. Although mTOR activity was increased in both human and animal kidneys with diabetic nephropathy, genetic deletion of mTORC1 in mouse podocytes induced proteinuria and progressive glomerulosclerosis. Furthermore, simultaneous deletion of both mTORC1 and mTORC2 from mouse podocytes aggravated the glomerular lesions. These results revealed the requirement for tightly balanced mTOR activity in podocyte homeostasis [128]. Therefore, it is important to test if reduction of podocyte mTOR activity can be harnessed as a potential therapeutic strategy to treat diabetic nephropathy [129].

8.2. Calcium Signaling and Proteinuria. Calcium ions are important mediators of cellular homeostasis owing to their ability to elicit a dynamic, transient, and tightly regulated range of biochemical responses [130]. The increase of the cytosolic Ca^{2+} activity may be an early event in the pathogenesis of protamine sulfate-mediated retraction of podocyte foot processes [131]. Previous studies had established an intimate association between Ca^{2+} influx and the activation of the Rho GTPases, which are cytoskeleton master regulators [132]. Recent study showed that Rho A played an important role in maintaining the integrity of the glomerular filtration barrier under basal conditions, but enhancement of Rho A activity above basal levels promoted podocyte injury

[133]. Moreover, activation of the Ca^{2+}-dependent phosphatase calcineurin leads to cathepsinL-mediated cleavage of synaptopodin and to proteinuria. The calcineurin inhibitor cyclosporine A (CsA) blocks the calcineurin-mediated dephosphorylation of synaptopodin and protects synaptopodin from cathepsin L-mediated degradation [134]. Recently, inhibition of calcium channels and chelation of extracellular calcium reduced protamine sulfate-induced foot process effacement and albumin leakage in rat model, and calcineurin inhibitors and the cathepsin L inhibitor all inhibited protamine sulfate-mediated barrier changes. All these results suggested calcium signaling played a critical role in the initial stages of glomerular injury and calcium signaling might act through calcineurin- and cathepsin L-dependent manner [135].

9. Conclusion

Renal proteinuria is a manifestation of glomerular filtration barrier dysfunction. It is currently under debate as to which layer of the glomerular filtration barrier plays the most important role in the pathogenesis of proteinuria. Moreover, the functions of some SD molecules remain to be elucidated. The studies described here, however, provide clues for future investigations into the pathogenesis of proteinuria, paving the way for and providing new ways think about treatment options for patients with proteinuria-related diseases.

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Review Article

Is There a Role for Mammalian Target of Rapamycin Inhibition in Renal Failure due to Mesangioproliferative Nephrotic Syndrome?

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Primary glomerulonephritis stands as the third most important cause of end-stage renal disease, suggesting that appropriate treatment may not be as effective as intended to be. Moreover, proteinuria, the hallmark of glomerular damage and a prognostic marker of renal damage progression, is frequently resistant to thorough control. In addition, proteinuria may be the common end pathway in which different pathogenetic mechanisms may converge. This explains why immunosuppressive and nonimmunosuppressive approaches are partly not sufficient to halt disease progression. One of the commonest causes of primary glomerulonephritis is mesangioproliferative glomerulonephritis. Among the triggered intracellular pathways involved in mesangial cell proliferation, the mammalian target of rapamycin (mTOR) plays a critical role in cell growth, in turn regulated by many cytokines, disbalanced by the altered glomerulopathy itself. However, when inhibition of mTOR was studied in rodents and in humans with primary glomerulonephritis the results were contradictory. In light of these controversial data, we propose an explanation for these results, to dilucidate under which circumstances mTOR inhibition should be considered to treat glomerular proteinuria and finally to propose mTOR inhibitors to be prospectively assessed in clinical trials in patients with primary mesangioproliferative glomerulonephritis, for which a satisfactory standard immunosuppressive regimen is still pending.

1. Introduction

The universal and growing impact of chronic diseases is undoubtedly high. While there has been little attention paid to kidney disease on a public health level, the reality is that many countries hardly bear the costs of providing end-stage renal disease care through renal replacement therapy. According to the latest USRDS report, while the prevalence of diabetes has clearly increased and the prevalence of congestive heart failure has remained stable, the prevalence of chronic kidney disease appears to have declined slightly in 2009, from 15.8 percent to 15.1 percent when calculated with the MDRD-4 formula and from 14.7 percent to 14.5 percent when calculated with the CKD-EPI formula; prevalence estimates of chronic kidney disease in USA in 1988–1994 had been 12.8 and 12 percent, respectively [1]. Obviously, differences in the prevalence estimates may in part differ depending on the criteria and equations employed. Among the most frequent causes of end-stage renal disease, glomerulonephritis ranks third worldwide.

Mesangioproliferative glomerulonephritis, mostly IgA nephropathy, is the most frequent primary glomerular disorder worldwide, and progressive mesangioproliferative nephropathy constitutes a major cause of end-stage renal disease [2, 3]. Recent long-term controlled studies assessing the prognosis have shown that the condition does not represent a benign disorder as previously mentioned [4–7]. Once renal function impairment develops, end-stage renal disease appears inevitable, often in the long term. Over 25 years of followup, about 30–50% of patients with IgAN will enter renal replacement therapy [8, 9]. Other causes of mesangial glomerulonephritis consist of pure mesangial

proliferative glomerulonephritis, IgM glomerulonephritis, and C1q glomerulonephritis [10].

Progression in mesangioproliferative glomerulonephritis generally involves increasing proteinuria, pathological renal extracellular matrix protein accumulation, cell proliferation, and inflammatory cell infiltration [11–15]. Pharmacological treatment of progressive mesangioproliferative disease is practically limited to renin-angiotensin system inhibition using angiotensin-converting enzyme inhibitors or type 1 angiotensin receptor antagonists, which serves to decelerate but is generally not capable of halting the advance of the disease [2, 13, 15]. As recently remarked by Floege and Eitner, there are few randomized controlled trials for IgA nephropathy and very rarely do patient numbers exceed 200. Consequently, most guidelines relating to IgAN are based on a low-to-very-low level of evidence and, in many cases, suggestions cannot even be offered. Thus, the majority of patients will continue to be treated based largely on opinion [16]. With respect to the other causes of mesangioproliferative glomerulonephritis, in the absence of controlled trials, it is difficult to determine the efficacy of therapy [10].

Administration of various immunosuppressive regimes, containing steroids and cytotoxic/cytostatic drugs, is increasingly adopted. Since there is no final agreement regarding the antiproteinuric efficacy of these protocols, which also vary in different disease states, and side effects are important, the clinical efficacy of new immunosuppressors is increasingly assessed [17–19]. Among these, the mammalian target of the rapamycin (mTOR) inhibitor family, which includes sirolimus and everolimus, is frequently used immunosuppressant with proliferation signal inhibitors properties employed in nephrology [17–20]. However, serious side effects including renal injury and proteinuria have been described during treatment with these drugs, in the setting of renal allograft nephropathy, human glomerulonephritis as well as in experimental kidney disease [19, 21–23]. Despite this, mTOR inhibition has been shown to be beneficial in chronic mesangioproliferative nephropathy and to reduce proteinuria in an experimental anti-Thy1 nephritis (a chronic model of progressive mesangioproliferative nephropathy) and in focal segmental glomerulosclerosis [24].

Aberrant proliferation of mesangial cells is a common finding in a number of diseases that can lead to end-stage renal failure. A variety of initial insults, which may be metabolic (as in diabetic nephropathy) or immunological (as in IgA disease and lupus nephritis), can cause uncontrolled mesangial cell proliferation. This in turn causes an increase in extracellular matrix deposition, ultimately leading to glomerulosclerosis, with subsequent activation of common intermediate pathways, associated with increased synthesis and a release of growth factors, cytokines, chemokines, and oxidant species, which stimulate the further proliferation of mesangial cells and finally mediating the damage of the kidney parenchyma. It is important to understand the proliferation mechanism of mesangial cell in order to determine the key regulatory molecular pathways involved in the pathological response to injury and ultimately to identify potential targets for therapeutic intervention [25].

2. Abnormal Cellular Mechanisms of Mesangial Proliferation in Mesangioproliferative Glomerulonephritis

Platelet-derived growth factor (PDGF) has been recognized as a major mitogen and one of the most important growth factors, which mediates multiple cellular activities such as cell proliferation, hypertrophy, and extracellular matrix protein synthesis in various types of cells including mesangial cells, and plays an important role in the changes in glomerular morphology in diabetic nephropathy [25, 26] and mesangioproliferative glomerulonephritis, mainly IgAN [27] (Figure 1). In recent years, growing evidence suggests that adiponectin is also an important regulator of cell proliferation. The data about its influence on cell proliferation are conflicting. Some authors suggest that adiponectin stimulates endothelial cell growth and angiogenesis [28, 29], whereas others perceive it as a negative regulator of cell proliferation [30, 31]. Su et al. found that adiponectin inhibited PDGF-induced mesangial cell proliferation. Mechanistic insights into this phenotype suggest that adiponectin attenuates PDGF-induced phosphorylation of mTOR via AMPK activation [25]. Interestingly, in a recent study it has been reported that in subjects with mesangial cell proliferation, as IgAN and diabetic nephropathy, there exists an increase of urinary adiponectin levels, which may partly result from enhanced filtration of circulating adiponectin through the changes of glomerular permselectivity and intraglomerular hydraulic pressure [32].

3. Regulation of mTOR

The mammalian target of rapamycin plays a critical role in cell differentiation, migration, and survival [33–35]. mTOR is an intracellular serine/threonine kinase and a central component of a complex signaling network that is highly conserved in evolutionary terms and expressed ubiquitously throughout the cells of the body. mTOR is a component of two major intracellular signaling complexes (mTORC1 and mTORC2), that play different roles downstream. mTORC1 is activated by growth factors and amino acids and controls cellular proliferation, promoting processes such as DNA translation, RNA transcription, ribosomal biogenesis, and cell cycle progression [35]. Inhibitors of mTOR, such as rapamycin or everolimus, bind to an intracellular cytoplasmatic receptor, the FK506-binding protein-12. The complex formed then interacts and disrupts mTOR function and leads to cell cycle arrest in the G1 phase. In addition to blocking cell proliferation, mTOR inhibitors have been found to be anti-inflammatory, antifibrotic, antitumoral, and antifungal, which underscores the involvement of mTOR signaling in a wide range of cellular functions [33, 34, 36] (Figure 1). Thus, mTOR is essential for the proliferation of mesangial cells, as is the case of mesangioproliferative glomerulonephritis. In this regard, in vitro studies with mice mesangial cells under the effect of different concentrations of rapamycin showed that a remarkably low dose (0.01 ng/mL) inhibited both proliferation and type IV collagen production. However,

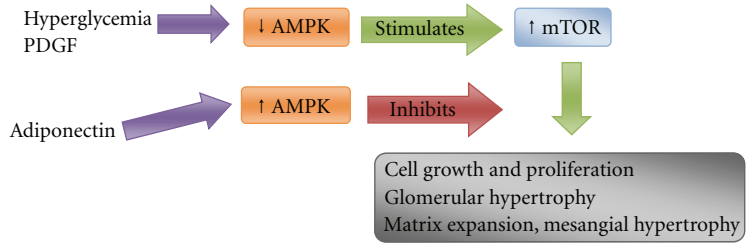

Figure 1: Hyperglycemia and PDGF stimulate mTOR, which in turn contribute to the nuclear translation of mRNAs necessary for cell growth and proliferation, clinically evident as hematuria, proteinuria, and glomerular filtration rate alterations.

this dose is below that used to produce clinically immunosuppressive effects (4.5–14 ng/mL), but could also minimize other potential adverse drug effects [37].

4. mTOR Inhibition: Low versus High Dose

In experimental studies, different doses of mTORi may play opposite effects on mesangial expansion. This may appear to be particularly true with respect to mesangioproliferative glomerulonephritis. In this respect, low-dose rapamycin (2.5 mg/kg^{-1}/body weight^{-1} in rats) confers antiproteinuric effects in a chronic model of progressive mesangioproliferative nephropathy, that is, anti-thy1-induced glomerulosclerosis in the rat [36]. Briefly, rapamycin remarkably limits the progressive course of chronic anti-thy1 antibody-induced renal disease towards glomerulosclerosis, tubulointerstitial fibrosis, and renal insufficiency. Renoprotection by low-dose rapamycin presents beneficial effects on a number of key pathways of renal disease progression, that is, proteinuria, renal matrix protein accumulation, cell proliferation, and leukocyte infiltration [12–15, 36]. Moreover, the beneficial effects of mTOR inhibition have recently been reported in several rat models of chronic kidney disease, that is, hypertensive 5/6 nephrectomy, diabetic nephropathy, hypertrophy following unilateral nephrectomy, tubulointerstitial fibrosis due to uretheral obstruction or nephrotic syndrome, and polycystic kidney disease, but not chronic transplant glomerulopathy [38–43]. Since these experimental studies represent the vast majority of human chronic kidney disease, their findings together imply that mTOR signaling acts as a rather common key pathway in the progression of renal disease. Furthermore, these studies suggest that inhibition of mTOR might be a novel, generally effective therapeutic approach to chronic kidney disease. However, the latter option may also apply to early preventive treatment, since this represents generally the timing of mTOR inhibition in experimental rat studies. However, the outcome of mTOR inhibition in anti-thy1-induced glomerulosclerosis contrasts with the one previously reported in anti-thy1-induced acute glomerulonephritis [44, 45]. In the latter, the mTOR inhibitor everolimus before or during the early marked mesangial cell proliferation turned out to be detrimental, manifesting aggravation of proteinuria, impaired self-healing, increased uremic mortality, and persistent glomerular fibrotic changes. These effects were observed both with high- and low-dose everolimus [44]. The studies in anti-thy1 acute and chronic renal disease unanimously indicate that unaffected mTOR signaling is critical for the very early and marked mesangial cell proliferation and subsequent normal glomerular repair of acute anti-thy1 glomerulonephritis [36, 44, 45]. In the further course of the disease, inhibition of mTOR even acts beneficially and prevents chronic disease progression [36]. Daniel et al. [45] reported renoprotective effect of sirolimus in experimental mesangioproliferative glomerulonephritis. Application of everolimus as late treatment for 14 weeks attenuated proteinuria and the time course of chronic anti-Thy1 nephritis in the rat, through reduction in vascular endothelial growth factor VEGF and TGF-β1expression [38].

VEGF is a potent mitogen expressed in podocytes and tubular cells that normally facilitates both glomerular and interstitial endothelial proliferation and angiogenesis [38, 46, 47]. Outlined by Schrijvers et al., the role of VEGF in normal renal physiology is essentially unknown [48]. However, VEGF and its receptors are upregulated in experimental animals and humans with type 1 and 2 diabetes and mesangioproliferative glomerulonephritis as IgAN [48–50]. Overexpression of VEGF-A leads to glomerular collapse, proteinuria, and end-stage renal disease in mice [50, 51]. In subjects with early IgAN, expression of VEGF is upregulated [52]. Some VEGF polymorphisms may be associated with the increased risk of renal progression in patients with IgA nephropathy [50]. Finally, VEGF expression is genetically regulated via the mTOR pathway [53, 54], which is in turn well known to be inhibited by both sirolimus and everolimus.

In contrast to Daniel et al. [44], Ramadan et al. have shown that either early or late low (20 mg/L) but not high doses (100 mg/L) of everolimus attenuated the loss of the slit diaphragm proteins nephrin and podocin in adriamycin-induced nephrotic syndrome (an experimental nephropathy that mimics minimal change disease), suggesting that the antiproteinuric effect of this agent at the therapeutic dose is due to preservation of nephrin and podocin. Specifically, chronic administration of everolimus at high therapeutic doses to normal rats did not induce renal injury as expressed by the lack of adverse effects on glomerular nephrin/podocin abundance. However, the authors speculate that this high-dose effect may be due to a decline of glomerular filtration rate and to the hypoalbuminemia seen in these rats [24].

The efficacy of mTORi in mesangioproliferative glomerulonephritis is limited. These potential beneficial results achieved in experimental studies are now expanding towards a progressive model of human mesangioproliferative nephropathy, an important cause of end-stage kidney disease

worldwide [2, 3]. However, clinical studies with sirolimus are scant and showed controversial results with other causes of glomerulonephritis. For example, while some studies reported a rapid decline in renal function with worsening proteinuria in patients with primary focal and segmental glomerulosclerosis [55, 56], others showed beneficial anti-proteinuric effects in this disease [39, 40]. However, with respect to mesangioproliferative glomerulonephritis and low-dose rapamycin, an interesting and provocative manuscript has recently been published. In this clinical study which included 25 subjects (15 on rapamycin), it has been shown that low-dose rapamycin plus angiotensin-converting enzyme inhibition (enalapril 5 mg/day) and statins (atorvastatin 10 mg/day) stabilized renal function and reduced glomerular proliferation in subjects with stage 3 chronic kidney disease measured with ^{51}Cr-EDTA technique due to IgA nephropathy with proteinuria >1 g/day. At 1 year, according to Oxford classification rapamycin treatment was associated with a significant reduction of mesangial and endocapillary proliferation. Moreover, patients receiving only angiotensin-converting enzyme inhibition and statins lost 8 mL/min/1.73 m^2, whereas those under rapamycin improved by 5 mL/min/1.73 m^2 ($P = 0.03$). However, sclerosis, chronic biopsy lesions, and proteinuria decreased similarly in both study groups [57]. With respect to proteinuria, baseline values dropped from 2.9 ± 1.8 to 2.0 ± 0.9 g/day at one year, compared with the respective decrease in the control group from 3.7 ± 1.6 to 2.8 ± 1.4 g/day. The dose of rapamycin employed was 1 mg/day with through levels between 4 and 8 ng/mL, as it is usually used in renal transplantation. Nevertheless, authors consider this rapamycin approach as a low-dose regime, as they compare it with a previous work by Fervenza et al. in which different classes of glomerulonephritis with proteinuria >1 g/day and creatinine clearance >20 mL/min were treated with rapamycin 5 mg/day to seek through levels between 7 and 10 ng/mL [23]. However, in this work renal function was measured with Cockcroft-Gault equation, only 5 of the 11 subjects included had IgA nephropathy, 6 developed acute renal failure presumably due to not-adjusted rapamycin levels according to renal function, and no data regarding initial or final proteinuria are offered [23]. Therefore, we believe both studies are not comparable, and the human "low-dose" mTORi for mesangioproliferative is still to be determined.

Finally, mTORi are not recommended in the transplanted patient when proteinuria exceeds approximately 800 mg/day. However, proteinuric mechanisms in this setting are not related to mesangial proliferation, which is the main culprit in mesangioproliferative glomerulonephritis. In addition, low dose of everolimus or rapamycin in human is to be determined.

We believe that rapamycin and everolimus must not be considered indistinctly as therapeutic alternatives in glomerular diseases. Human doses have not been established, and randomized trials in humans are required before recommendations are made. Whether preferentially early versus late, low-versus-high doses of mTORi effects on proteinuria in mesangioproliferative GNs are due to VEGF reduced expression, to podocin-nephrin protection, and/or to interventions in the adiponectin-PDGF binomium, among many other possibilities, is a novel, interesting, and a promising field of research to assess treatment of acute mesangioproliferative glomerulonephritis, for which no specific immunosuppressive standardized therapy exists.

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Review Article

Mechanisms of Glomerular Albumin Filtration and Tubular Reabsorption

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Albumin is filtered through the glomerulus with a sieving coefficient of 0.00062, which results in approximately 3.3 g of albumin filtered daily in human kidneys. The proximal convoluted tubule reabsorbs 71%, the loop of Henle and distal tubule 23%, and collecting duct 3% of the glomerular filtered albumin, thus indicating that the kidney plays an important role in protein metabolism. Dysfunction of albumin reabsorption in the proximal tubules, due to reduced megalin expression, may explain the microalbuminuria in early-stage diabetes. Meanwhile, massive nonselective proteinuria is ascribed to various disorders of the glomerular filtration barrier, including podocyte detachment, glomerular basement membrane rupture, and slit diaphragm dysfunction in focal segmental glomerulosclerosis, membranous nephropathy, and other glomerulonephritis. Selective albuminuria associated with foot process effacement and tight junction-like slit alteration is observed in the patients with minimal-change nephrotic syndrome, and the albumin uptake is enhanced in the podocyte cell body, possibly mediated by albumin receptors in the low-dose puromycin model. The role of enhanced podocyte albumin transport needs to be investigated to elucidate the mechanism of the selective albuminuria in minimal-change disease.

1. Introduction

The kidneys are responsible for maintaining the homeostasis of body fluids by the regulation of water balance, electrolyte balance, acid-base balance, and excretion of uremic toxins, and also production of various hormones such as renin, erythropoietin, and activation of vitamin D_3. However, little attention has so far been paid to the role of protein metabolism by the kidney. Primitive urine filtered by the glomerulus contains many proteins smaller than albumin, and the renal proximal tubules actively reabsorb these proteins, which are subsequently degraded to amino acids in lysosomes and returned to the blood [1]. This paper describes the mechanisms and pathways of glomerular albumin filtration and the amount of tubular reabsorption of albumin along the nephron in normal and pathological conditions based on our previous micropuncture studies. The concept that glomerular albumin filtration is restricted by the size and charge barriers of the glomerular basement membrane, and finally by the fine pores of the slit diaphragm, is widely accepted. However, Smithies [2] raised an essential issue; why do the slit diaphragms not "clog" with albumin if all filtered albumin molecules pass through them? Although glomerular albumin filtration could be performed by the diffusion of albumin back and forth across the GBM [3], how albumin molecules can diffuse out across the effaced podocyte foot processes entirely covering the basement membrane in minimal-change nephrotic syndrome remains unclear. This paper discusses the ultrastructural morphological changes of the glomerular filtration barrier in various glomerular diseases and proposes a new mechanism of glomerular albumin filtration in minimal-change nephrotic syndrome.

2. Albumin Filtration by the Glomerulus under Normal Conditions

Albumin contains three spherical domains, with a molecular weight of 69 kDa and a net charge of −15. It is a flexible,

Table 1: Comparison of the glomerular albumin-sieving coefficient (SC) values.

Authors, year	Method	SC	Species/animal model
Tojo and Endou [12], 1992	Fractional micropuncture	0.00062	rat
Bertolatus and Hunsicker [62], 1985	^{131}I-labeled BSA measuring urinary excretion and total kidney uptake minus interstitial nonfiltration uptake	0.0006	rat
		0.021	hexadimethrine nephrotic rat
		0.025	adriamycin nephrotic rat
	^{131}I-labeled neutral BSA	0.026	rat
Lund et al. [18], 2003	^{125}I-native human serum albumin measuring both kidney uptake and urinary excretion	0.00066	rat
	^{125}I-neutral human serum albumin	0.0065	rat
Norden et al. [13], 2001	urinary albumin excretion of congenital Fanconi syndrome patients	0.00008	human
Tencer et al. [63], 1998	Blockade of proximal tubular reabsorption by L-lysine	0.00033	rat
		0.0591	puromycin aminonucleoside nephrotic rat
Ohlson et al. [64], 2000	Inhibition of tubular function by cooling (8°C)	0.0019	rat
Christensen et al. [23], 2007	urinary albumin excretion of megalin-knockout mice	0.00016	megalin-knockout mice
Eppel et al. [14], 1999	tritium-labeled albumin	0.074	rat
Russo et al. [15], 2007	Alexa-labeled albumin, confocal microscopy	0.0341	rat
Tanner [16]	Alexa-labeled rat serum albumin, two-photon microscope with internal photodetectors	0.002	Munich-Wistar rat

ellipsoid-shaped molecule, 3.8 nm in diameter and 15 nm long molecule [1, 4]. The slit pore size was originally reported to be a rectangular pore approximately 40 by 140 Å in cross section and 70 Å in length [5], and as a recent electron tomography study revealed, the glomerular slit-pores are 35 Å (3.5 nm) in diameter with some variation in size [6]. These measurements were performed on samples processed for electron microscopy, in which a slight reduction in size is inevitable, so the true size of these pores are likely to be larger than these values. Although the effective Stock-Einstein radius of albumin is 35 Å (70 Å in diameter), some albumin molecules are able to pass through the slit pores, due to their flexibility and ellipsoid shape. This is consistent with the observation of FITC-labeled albumin on the slit diaphragms between foot processes, indicating that a small fraction of albumin could pass through the slit pores in normal rats [7]. Early micropuncture studies demonstrated albumin concentration values form 3 to 728 μg/mL in primitive urine in Bowman's capsule in normal rats [8–11]. The large variation has been interpreted as contamination with albumin in the serum from the peritubular capillaries during the collection of tubular fluid. Therefore, the fractional micropuncture method was developed to avoid serum albumin contamination. Renal tubules are initially punctured with an outer pipette, and then four fractions of tubular fluid are collected with an inner pipette. The albumin concentration is measured in the fourth fraction of tubular fluid, which is virtually free from contamination by serum albumin, yielding a value of 22.9 μg/mL in Bowman's capsule, and an albumin-sieving coefficient of 0.00062 [12].

Isotope-labeled albumin clearance studies, which measured both urinary excretion and tubular uptake, divided by the plasma isotope level, showed consistent values with our fractional micropuncture data (Table 1). Albumin clearance studies after blocking proximal tubular reabsorption with L-lysine, treatment with low temperatures, or studies of congenital abnormalities of tubular reabsorption showed slightly smaller values in comparison to the micropuncture data.

The fractional excretion of albumin in Fanconi syndrome patients is 0.00008, and this may be approximately equivalent to the glomerular-sieving coefficient in the normal kidney [13]. However, nephron segments downstream of the proximal convoluted tubules can reabsorb about 26% of glomerular filtrated albumin even if proximal tubular albumin reabsorption is impaired in Fanconi syndrome, (Figure 1). Therefore, the glomerular albumin-sieving coefficient may actually be greater than 0.00011 in humans.

Larger amounts of glomerular albumin filtration have been reported. One study using tritium-labeled albumin demonstrated a sieving coefficient of 0.074 [14], and another study using Alexa-labeled albumin observed by confocal microscopy resulted in a sieving coefficient of 0.0341 [15], which is 50 to 100 times higher values than previous studies. However, there were several technical limitations including the sensitivity of the measurement methods, the interference by out-of-focus fluorescence, and the incomplete removal of unbound labeling molecules, which can freely pass the glomerular filtration barrier. These values also seem unrealistically high from the viewpoint of albumin metabolism.

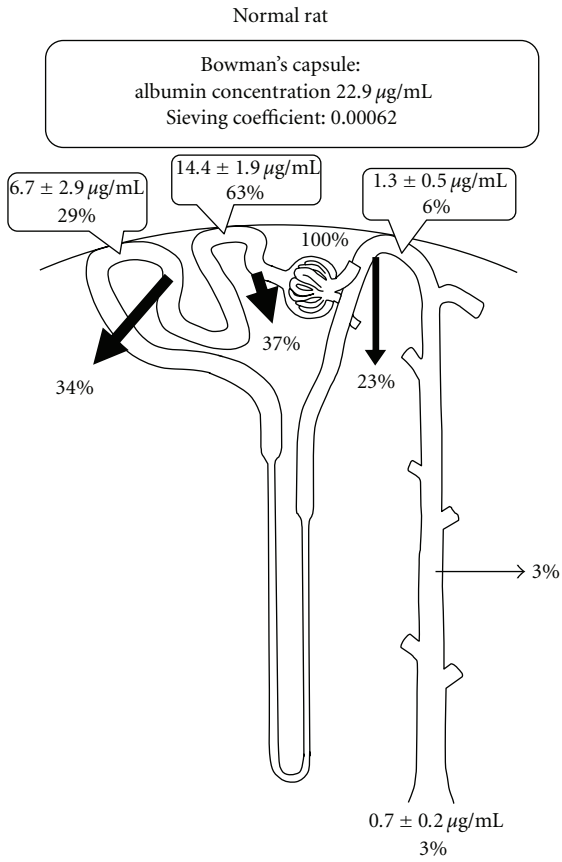


Figure 1: Albumin concentration along the nephron calculated from the data from a rat fractional micropuncture study [12].

The problem of out-of-focus fluorescence contaminating the signal from Bowman's capsule was solved by performing two-photon microscopy studies utilizing internal photodetectors, and the glomerular-sieving coefficient was calculated as 0.002 with Alexa fluor labeled-rat serum albumin [16] and 0.001 with Rhodamine labeled 70-kD dextran [17], which are closer to the values estimated from micropunctures.

The glomerular-sieving coefficient is not a static constant parameter. The value changes in response to oscillational changes in GFR, temperature or laparotomy during experiments [17–20], and it may vary in the range from 0.0001 to 0.0006 under normal conditions.

3. The Important Role of the Kidney in the Protein Metabolism

Albumin concentration along the rat nephron was measured in fractional micropuncture studies (Figure 1) [12]. Renal tubules reabsorb about 3 g of albumin per day in humans (Table 2). The albumin reabsorption capacity measured in the isolated rabbit proximal tubule was 99.9×10^{-3} ng/min/mm [21]. The length of the proximal tubule is 6.5 mm [22], so human kidneys can be estimated to reabsorb 1.9 g (99.9×10^{-3} ng/min/mm $\times$ 24 h $\times$ 60 min $\times$ 6.5 mm $\times$ 2 $\times$ 10^6 nephron) of albumin per day. The nephron segments downstream of the proximal convoluted tubules further reabsorbs about 26% of filtered albumin, thus the total amount of albumin reabsorption in the kidney comes to 2.6 g a day, which is consistent with the micropuncture data. Albumin molecules are taken up into lysosomes in the proximal tubule within 6 to15 minutes and then degraded to amino acids after 30 to 120 minutes in the proximal tubule [1, 21]. Therefore, the kidney should be regarded as an organ that plays an important role in the protein metabolism.

The high sieving coefficient values reported by Russo et al. [15], which suggest that about 200 g of albumin per day are filtered in the glomerulus and reabsorbed in the proximal tubule, seem highly unlikely. In fact, abundant amounts of albumin cannot be detected in the normal kidney by immunostaining. In addition, the proximal tubule is unlikely to be able to transport such huge amounts of intact albumin under physiological conditions [21, 23, 24]. It is unreasonable that albumin filtration and metabolism in the kidney is larger than the daily production by the liver (~20 g), or than the total plasma albumin (~125 g).

On the other hand, low molecular weight proteins are almost all freely filtered at the glomerulus with a sieving coefficient of 0.987 [12] and about 9.6 g are reabsorbed per day (Table 2). The tubular dysfunction of protein metabolism in chronic renal failure cannot be compensated for by hemodialysis, so low molecular weight protein deposits, such as β2-microglobulin, in various organs, cause amyloidosis in hemodialysis patients. The physiological role of protein metabolism in the kidney must be accounted for.

4. Mechanism of Microalbuminuria in Diabetic Nephropathy

Microalbuminuria is an early marker of diabetic nephropathy and is believed to occur due to increased glomerular permeability and glomerular hyperfiltration [25, 26]. However, a fractional micropuncture study demonstrated the proximal tubular albumin reabsorption to decrease without an increase in the glomerular albumin filtration in the early stages of streptozotocin-induced diabetic nephropathy (Figure 2) [27]. This tubular dysfunction may be one of the mechanisms of microalbuminuria in the early-stage diabetes. This is supported by the finding that megalin, the receptor for albumin endocytosis in the proximal tubules, is decreased in diabetic rats [27], and by the measurement of albumin clearance after the blockade of proximal reabsorption with lysine, utilizing isotope labeled-bovine serum albumin [28]. There is a possibility that the true amount of albuminuria may be larger than that detected in the urine by measuring the intact albumin, because albumin degrades to fragmented albumin by brush border enzymes in the proximal tubules, [29].

Renin-angiotensin system (RAS) inhibitors are reported to restore megalin expression, ameliorate the tubular dysfunction of albumin reabsorption, and reduce albuminuria in diabetic rats [30]. Albumin is reabsorbed by receptor-mediated endocytosis into endosomes, where ligand-receptor dissociation must occur to recycle the

Table 2: Glomerular filtration and reabsorption of albumin and low-molecular weight proteins (LMWP) in humans estimated from rat micropuncture data [12].

	Albumin	LMWP
Bowman's capsule concentration	22.9 μg/mL	72.1 μg/mL
Plasma concentration	37.0 mg/mL	73.0 μg/mL
Sieving coefficient	0.00062	0.987
Estimated glomerular filtration amount in humans	22.9 μg/mL $\times$ 100 mL/min $\times$ 24 h $\times$ 60 min = 3.3 g/day	72.1 μg/mL $\times$ 100 mL/min $\times$ 24 h $\times$ 60 min = 10.4 g/day
Estimated tubular reabsorption in humans	3.2 g/day	9.6 g/day

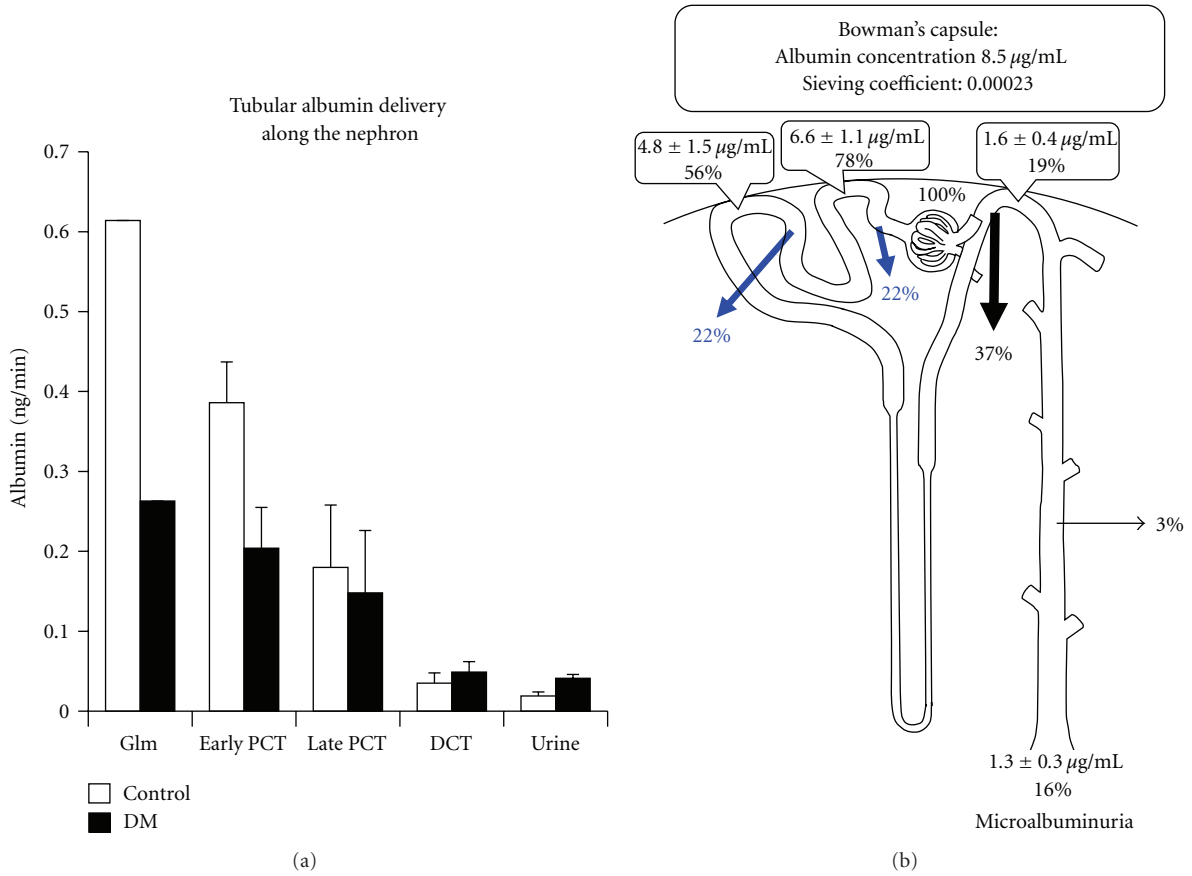
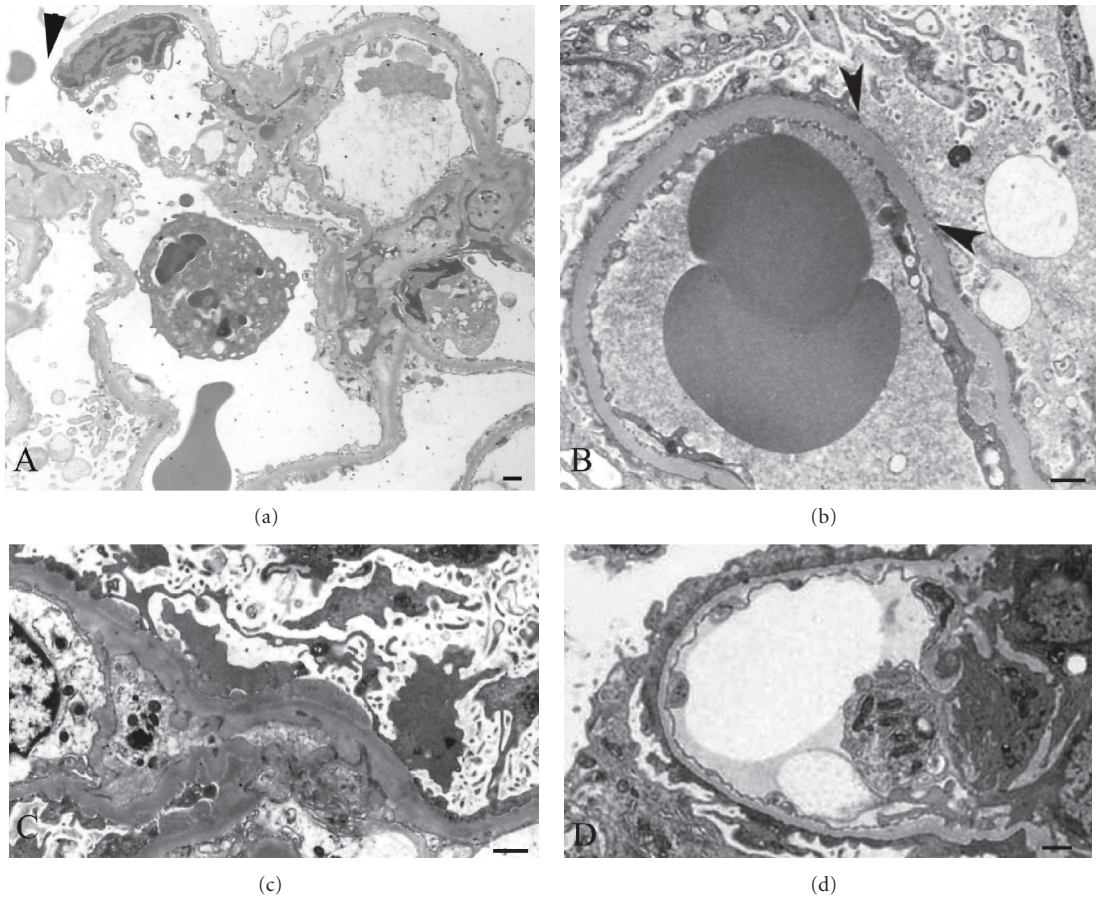


Figure 2: Tubular albumin reabsorption in diabetic nephropathy calculated from the data from a rat fractional micropuncture study. Albumin reabsorption in the proximal convoluted tubules (PCT) is significantly reduced in diabetes mellitus (DM) rats, resulting in higher albumin delivery in the distal convoluted tubules (DCT) and urine in DM rats than in normal controls.

albumin-binding receptors back to the plasma membrane. Vesicular acidification by H^+-ATPase, CLC-5, NHE-3 is functionally important for the pH-dependent dissociation between albumin and megalin, and effective albumin reabsorption [1, 31]. Renal tissue angiotensin II levels are elevated in diabetes [32]. Angiotensin II blocks H^+-ATPase [33], thus acidification of endosomes may be reduced by inhibition of H^+-ATPase by renal angiotensin II, thus leading to decreased albumin reabsorption. Therefore, RAS inhibitors not only prevent intraglomerular hypertension and disrupt of glomerular permselectivity [34], but also restore albumin metabolism in the proximal tubules.

5. Questions Pertaining to Glomerular Albumin Filtration through Slit Pores in the Nephrotic Syndrome

The glomerular filtration barrier is made up of three layers; (1) the fenestrated endothelium covered by a negatively charged glycocalyx, (2) the glomerular basement membrane, where a size barrier is containing laminin and type IV collagen and a charge barrier generated by heparan sulfate is presumed to function as a coarse barrier, (3) and finally the slit diaphragm between foot processes, which is regarded as a fine filter [35]. Yamada identified the slit diaphragm



(a) (b)

(c) (d)

Figure 3: Electron microscopy of human renal biopsy samples. (a) Glomerular basement membrane rupture (arrowhead) in IgA nephropathy. (b) Podocyte loss and a denuded glomerular basement membrane in focal segmental glomerulosclerosis. Proteins were observed as nonuniform electron dense substances in the capillary lumen and also in the urinary space adjacent to the denuded glomerular basement membrane (arrow heads), suggesting large amounts of protein including albumin filtered through the denuded glomerular basement membrane, leading to proteinuria. (c) Podocyte detachment and apoptosis in membranous nephropathy with subepithelial electron dense deposits. (d) Diffuse foot process effacement with reduction of the slit membranes in minimal-change nephrotic syndrome. Bars indicate 500 nm.

by electron microscopy in 1955 [36] and Rodewald and Karnovsky identified the zipper-like structure of the slit membrane [5], indicating that albumin is filtered through the slit pores, similar to water, ions, and low molecular proteins. Experiments using various tracers raised the controversy over whether the GBM or the slit diaphragm is the most crucial restrictive filtration barrier; ferritin accumulates in the GBM, but not under the slit diaphragm [37], whereas horseradish peroxidase (HRP) is observed on the slit diaphragm as well as on the GBM [38, 39]. The identification of nephrin at the slit diaphragm and its mutation in the Finnish-type congenital nephrotic syndrome provided crucial evidence that the slit membrane is the main component of the glomerular filtration barrier [40, 41]. However, the importance of the GBM cannot be ignored, for laminin $\beta 2$ (LAMB2) knockout mice show a severe disorganization of the GBM structure, and accompanying proteinuria [42]. In addition, the importance of the charge barrier of GBM needs to be further investigated. Heparan sulfate synthase deficient mice do not show proteinuria even though they show reduction of the negative charge of the GBM and foot process effacement [43].

Therefore, both the slit diaphragm and the GBM are important for a functioning glomerular filtration barrier. However, several critical questions need to be answered before accepting the concept of albumin filtration through slit pores, (1) albumin does not accumulate under the slit diaphragm in tracer studies, (2) the sieving coefficient of albumin is much lower than Dextran or Ficoll of similar size, which cannot be fully explained from differences in flexibility of the molecular shape and charge between these molecules [4, 44], (3) the glomerular sieving coefficient to albumin should increase at high glomerular filtration rates, if the slit pores are the main barrier for albumin restriction; however, the opposite phenomenon has also been reported [18], (4) the number of slit diaphragms decreases with a tight junction like structural change in minimal-change nephrotic syndrome, and (5) reduction of nephrin may cause enlargement of the slit pores, that can explain massive proteinuria but not selective albuminuria in minimal-change

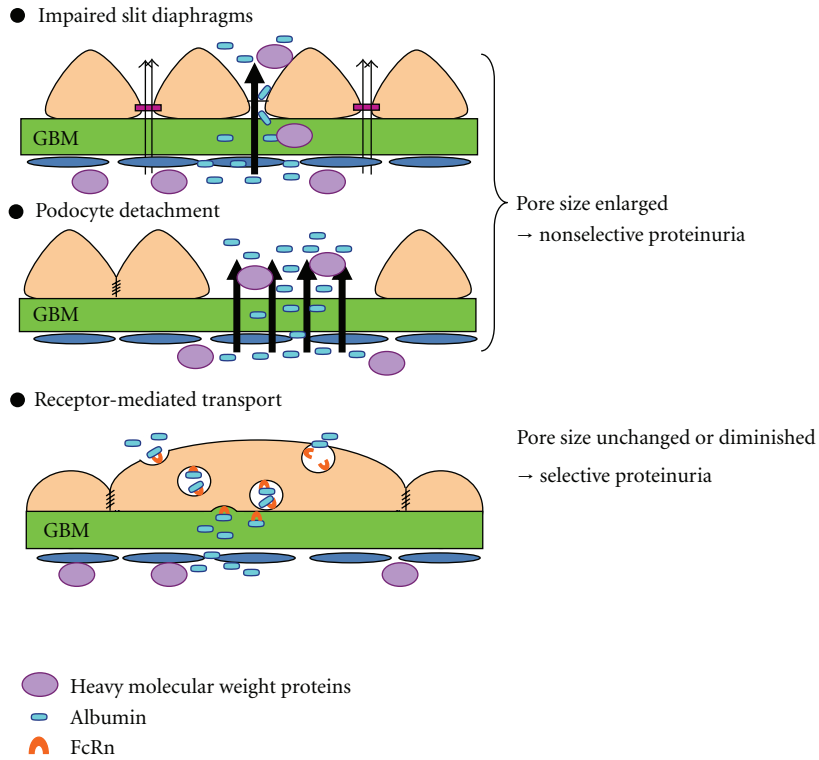


Figure 4: Possible mechanisms of albuminuria in minimal-change nephrotic syndrome. Albumin is filtered through the endothelial fenestrae, the basement membrane, and finally through the impaired slit diaphragm. Albumin is also filtered through the glomerular capillary wall where podocytes are lost by podocyte detachment or apoptosis causing enlarged slit pores and nonselective proteinuria. A mechanism of receptor-mediated albumin transport via FcRn through podocytes may explain the selective albuminuria in minimal-change disease.

nephrotic syndrome. It is noteworthy that both HRP and ferritin tracers are identified in the podocyte cytoplasm in some tracer studies [37, 39]thus suggesting that some transport mechanism may also exist for albumin.

6. Electron Microscopic Observation of Various Mechanisms of Proteinuria in Glomerular Diseases

Careful observation of human renal biopsies in electron microscopy images makes it possible to identify lesions responsible for proteinuria in various glomerular diseases (Figure 3). Glomerulonephritis including IgA nephropathy, ANCA-related nephritis, acute glomerulonephritis, and lupus nephritis show damage of the GBM by inflammatory cells forming ruptures or holes causing nonselective proteinuria with accompanying hematuria (Figure 3(a)). Podocyte detachment and podocyte apoptosis are observed in focal segmental glomerulosclerosis and membranous nephropathy, and proteins leak from the site of denuded GBM, causing nonselective proteinuria (Figures 3(b) and 3(c)). The denuded GBM is prone to adhere to the Bowman's capsule, resulting in segmental sclerosis. Podocyte detachment or apoptosis could occur by various mechanisms including hemodynamic stretching, immunological mechanisms such as immune complex deposition, integrin-dependent signaling, and oxidative stress derived from NADPH oxidase following stimulation by angiotensin II and cytokines [45–49]. These ultrastructural morphological changes of the GBM may represent the shunt pathway assumed in mathematical models of glomerular permselectivity [3, 50]. While these findings are often observed in focal segmental glomerulosclerosis and the "high dose" puromycin aminonucleoside nephrotic syndrome model [50], such shunts are rarely observed in both human minimal-change nephrotic syndrome and the low-dose puromycin model.

7. Possible Mechanisms of Selective Albuminuria in Minimal-Change Nephrotic Syndrome

Possible mechanisms of proteinuria in minimal-change nephrotic syndrome are schematically shown in Figure 4. The common assumption is that proteins leak from the slit pores due to reduced nephrin expression, while podocyte detachment is rarely observed [51, 52]. Podocytes with effaced foot processes widely cover the glomerular capillary wall in minimal change nephrotic syndrome (Figure 3(d)), and podocyte slit pore density is decreased by 80% at most, and half of the slits display a tight-junction-like structure [51]. These structural changes in the podocytes

raise the question of which route albumin actually passes through. Even if massive amounts of albumin are filtered through the altered slit membrane, it is difficult to explain the selective proteinuria by decrease in nephrin, which should lead to enlarged slit pores. In addition, there is discrepancy in time between the peaks of proteinuria and the expression of nephrin in the nephrotic model induced by an antibody against nephrin [53, 54] and in the puromycin aminonucleoside model [55]. It is possible that reduced nephrin expression is not a cause of nephrotic syndromes, but merely a reflection of the decrease in slit pore number. The results of labeled albumin tracer studies suggest that albumin may be transported through the podocyte cell body by endocytosis and exocytosis [7]. This hypothesis was confirmed using Evans blue (EB, molecular weight 961 Da) labeled albumin, which strongly binds to albumin without altering albumin's molecular weight, and shows red fluorescence, in the puromycin nephrotic model in GFP transgenic rats [56]. Podocytes emitting green GFP fluorescence turn yellow after the uptake of EB-labeled albumin. There is an initial delay of approximately 5 minutes before appearance of EB-albumin in the tubular lumen, which may be due to the time needed for the transcellular transport of albumin [56]. There are several receptors for albumin including megalin and cubilin in the proximal tubule [57], gp60 in the endothelium [58], and FcRn in podocytes [59, 60]. Interestingly, blocking the FcRn receptor with an antibody for FcRn reduces proteinuria, thus suggesting that the transport of albumin in the podocyte is at least partially mediated by FcRn [56]. The capacity for albumin endocytosis is large enough to explain daily albumin filtration through the podocytes (V_{max} 97.4 μg/mg cell protein/h) [61]. This value indicates that the estimated total endocytic capacity in human kidneys is 3.6 g/day [97.4 μg/mg cell protein/h $\times$ 0.21 mg cell protein/mg cell $\times$ cell volume $(4/3)\pi \times (10\,\mu\text{m})^3 \times$ 878 podocytes per glomerulus $\times$ 2 $\times$ 10^6 glomeruli per kidney $\times$ 24 h].

These findings shed new light on the possibility of filtration pathways of albumin through the podocyte cell body via receptor-mediated transcytosis. This mechanism of albumin filtration may answer the essential question raised by Smithies [2] of why the slit diaphragms do not "clog" with albumin in nephrotic syndrome. Further studies are necessary to elucidate how albumin is transported and excreted through the podocyte cell body.

8. Conclusion

In conclusion, the glomerular-sieving coefficient of albumin is 0.00062, and the kidney plays an important role in protein metabolism. Tubular dysfunction of albumin endocytosis via megalin can explain the microalbuminuria in the early-stage diabetic nephropathy. The identification of nephrin indicated that the slit diaphragm with slit pores may be a restriction filter for albumin molecules. Podocyte detachment and apoptosis or GBM rupture may explain the nonselective proteinuria with or without hematuria. Meanwhile, selective albuminuria in minimal-change nephrotic syndrome may be explained by the receptor-mediated transcytosis of albumin by podocytes, and this could be a new target for the treatment of the nephrotic syndrome.

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Review Article

Urinary Markers of Glomerular Injury in Diabetic Nephropathy

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Diabetic nephropathy, the leading cause of renal failure worldwide, affects approximately one-third of all people with diabetes. Microalbuminuria is considered the first sign and the best predictor of progression to renal failure and cardiovascular events. However, albuminuria has several limitations. Therefore, earlier, more sensitive and specific biomarkers with greater predictability are needed. The aim of this paper is to discuss the current literature on biomarkers of glomerular injury that have been implicated in diabetic kidney disease.

1. Introduction

Diabetes mellitus is a chronic disease that affects 366 million people worldwide (6.4% of the adult population) and is expected to rise to 552 million by 2030 [1]. People with diabetes require at least two to three times the health-care resources compared to people who do not have diabetes, and diabetes care may account for up to 15% of national health care budgets [2]. In 2008, 1.3 million deaths were associated with diabetes [3].

Diabetes results in both microvascular and macrovascular complications. Among the microvascular complications, diabetic kidney disease is one of the most serious, with significant impact on morbidity, mortality, and quality of life [4]. Diabetic nephropathy occurs in approximately one-third of all people with diabetes and is the leading cause of renal failure in developed and developing countries [3]. Death due to renal disease is 17 times more common in diabetics than in nondiabetics [5].

Clinically, the first sign of diabetic nephropathy is considered to be microalbuminuria. As the disease progresses, patients develop macroalbuminuria, and the kidney function declines until patients end up requiring renal replacement therapy [6].

Although microalbuminuria in diabetic patients is considered to be the best predictor of progression to end-stage renal disease [7] and cardiovascular events [8–10]; earlier, more sensitive and specific markers of kidney damage might help diagnose and treat diabetic nephropathy at an earlier stage to prevent the progression to renal failure.

Diabetic nephropathy affects all the kidney cellular elements, that is, glomerular endothelia, mesangial cells, podocytes, and tubular epithelia [11]. It is characterized by excessive accumulation of extracellular matrix (ECM) with thickening of glomerular and tubular basement membranes and increased amount of mesangial matrix, which ultimately progresses to glomerulosclerosis and tubulointerstitial fibrosis [11–13]. Multiple biomarkers in serum and urine have been studied that represent different mechanisms or structural damage, based on which they have been classified as markers of glomerular injury, tubular injury, oxidative stress, inflammation, and endothelial damage [5, 12].

Urinary markers of glomerular damage represent either, increased permeability to plasma proteins (albumin; transferrin), or increased excretion of extracellular matrix proteins (type IV collagen; fibronectin) [13]. The former is secondary to three main factors: loss of glomerular charge selectivity [14–20], loss of glomerular size selectivity [16, 18, 21–24], or increased intraglomerular pressure [25–27]. This paper will review the literature available regarding urinary biomarkers of glomerular injury associated with diabetic nephropathy.

2. Albumin

Albumin, a 65-kDa protein produced in the liver, is the most abundant plasma protein in the body. The main functions of albumin are to regulate the oncotic pressure, to act as an acid/base buffer, and to mediate the transportation of metabolites, hormones, vitamins, and drugs [28].

In normal subjects, a small amount of albumin is filtered in the glomerulus, but almost all of it is reabsorbed by the tubules [29, 30]. Elevated urine albumin excretion (UAE) is considered a well-established marker of glomerular damage [12]. In addition, it is known that tubular dysfunction by itself may cause albuminuria owing to decreased reabsorption of filtered albumin [28].

The UAE is considered normal when it is less than 30 mg/day or 20 microg/min (normoalbuminuria). This threshold was determined because the UAE of 95% of "normal" patients falls below this value [8]. However, it has been recognized that the risk of cardiovascular events and renal morbidity is elevated also in subjects in the "high normal" range [8, 31–33].

Based upon the ability of dipstick to measure urine albumin, the UAE has been classified as microalbuminuria, when the UAE is between 30 and 300 mg/day or 20 and 200 microg/min; macroalbuminuria, when the UAE is above 300 mg/day or 200 microg/min. The rate of progression from micro to macroalbuminuria in type 2 diabetic patients is 2-3% annually [34].

Baseline albuminuria is the strongest predictor of end-stage renal disease (ESRD) for type 2 diabetic patients [7]. But, not all diabetic patients develop diabetic nephropathy. Approximately 20 to 40% of diabetic patients develop microalbuminuria within 10–15 years of diagnosis, whereas macroalbuminuria occurs within 15–20 years in 20–40% of patients [6]. The American Diabetes Association recommends screening with annual UAE on all type 1 diabetic patients with ≥ 5 years of disease duration and in all type 2 diabetic patients starting at diagnosis [35].

Microalbuminuria is not only a risk factor for chronic kidney disease (CKD) and ESRD, but it is also a strong predictor of total and cardiovascular mortality and cardiovascular morbidity in diabetic patients [8–10]. In patients with type 2 diabetes and nephropathy, albuminuria is the strongest risk marker for cardiovascular events [36].

Although albuminuria is widely used and is considered the best marker for renal damage in diabetic patients, certain limitations should be taken into consideration. First, not all patients with proteinuria will develop progressive renal dysfunction [6, 34]. Additionally, 30% of diabetic patients with renal impairment have normoalbuminuria [37]. Second, the cardiovascular and renal morbidity is elevated in the "high normal" range of UAE [8, 31–33]. Third, a number of variables affecting UAE lack standardization including urine collection methods, reporting of test results, reference intervals for albumin-to-creatinine ratio and lack of a complete reference system for urine albumin and creatinine measurements [38]. Finally, multiple markers of renal dysfunction, either tubular or glomerular, can appear before the detection of microalbuminuria, suggesting that microalbuminuria occurs once significant kidney damage has already occurred [12, 39].

3. Transferrin

Transferrin is a plasma protein very similar in weight (molecular weight 76.5 kDa) to albumin, but slightly larger (molecular radius 4.0 nm compared to 3.6 nm of albumin). It is less anionic than albumin with an isoelectric point (pI) one unit higher, therefore, expected to be filtered more readily through the glomerular barrier. Transferrin is the major iron-binding protein in the serum, and it transports ferric ions to all proliferative cells in the body [5, 12].

Among type 2 diabetic patients, urinary transferrin significantly increases with respect to the progress of biopsy proven glomerular diffuse lesions [40] and has been shown that some type 2 diabetic patients with diffuse glomerular lesions without microalbuminuria had microtransferrinuria [40]. Urinary transferrin excretion has also been correlated with the degree of interstitial fibrosis, tubular atrophy, and interstitial inflammatory cell infiltration [41].

Compared to healthy controls, transferrin excretion is higher in diabetic patients, even before they develop microalbuminuria [40, 42–48]. Because diabetic patients are more likely to have transferrinuria than albuminuria [44, 49–54], and because the albumin/transferrin ratio was significantly smaller in normoalbuminuric and microalbuminuric compared to macroalbuminuric patients, urinary transferrin is considered to be a more sensitive marker of glomerular damage in diabetic patients [44, 50–54]. Furthermore, increased urinary transferrin excretion predicts the development of microalbuminuria in type 2 diabetic patients with normoalbuminuria [55, 56]; in patients that already developed albuminuria, the urinary transferrin excretion has a linear relationship with UAE [39, 40, 42–44, 47, 50, 53, 57–60].

Urinary transferrin excretion is elevated in primary glomerulonephritis and other diseases that affect the glomerulus and is not specific to diabetic nephropathy [61, 62].

Although transferrinuria has been implicated as a cardiovascular risk factor, type 2 diabetic patients with both microalbuminuria and microtransferrinuria have a higher risk of ischemic heart disease than patients with microtransferrinuria only, suggesting that microalbuminuria may be a better predictor of ischemic heart disease than microtransferrinuria [60].

Urinary transferrin excretion is not correlated with glycemic control (hemoglobin A1c, fructosamine, and random glucose), supporting the hypothesis that transferrinuria is caused by intrinsic renal damage [42]. Nevertheless, glycemic control in newly diagnosed type 2 diabetic patients can effectively decrease transferrinuria [59]. Further evidence of transferrinuria as a marker of diabetic microvascular complications is the fact that urinary transferrin excretion is higher in type 2 diabetic patients with retinopathy [42, 44]. Conflicting results have been published regarding the correlation of urinary transferrin excretion and duration of diabetes [42, 44].

Similar to albumin [63], transferrin/creatinine ratio is associated with blood pressure control [42, 44]. However, only transferrinuria, and not albuminuria, has a correlation with diurnal changes in blood pressure [64].

In type 2 diabetic patients, transferrinuria precedes tubulointerstitial changes found on biopsy [41]. It is known that reabsorption of transferrin results in release of reactive iron [57], which can produce oxidative stress on the tubular epithelium. Several studies have shown that markers of proximal tubule damage (i.e., alpha-1-microglobulin and N-acetyl-beta-D-glucosaminidase (NAG)) and urinary transferrin excretion are associated in diabetic patients [40, 43, 44, 53, 59]. It is not clear if transferrinuria is secondary to decreased tubular reabsorption, or transferrin is the cause of tubular damage.

According to two small nonrandomized trials, low-dose angiotensin receptor blockers (ARBs) seem to prevent the progression of transferrinuria, or even reverse it, independent of their antihypertensive effect. But further randomized controlled trials are needed to support that conclusion [48, 65].

4. Type IV Collagen

Type IV collagen is the main constituent of both glomerular and tubular basement membranes as well as the mesangial matrix [5, 66]. Elevated glucose levels stimulate type IV collagen synthesis and may reduce its breakdown by producing advanced glycosylation of proteins. As a consequence, increased deposition of type IV collagen has been noted in the glomerular mesangial matrix of diabetic kidneys with diffuse glomerulosclerosis [5, 67, 68]. Additionally, urinary type IV collagen excretion has been associated with mesangial expansion and tubulointerstitial and glomerular injury [69, 70]. The urinary excretion of type IV collagen correlates with the urinary excretion of other components of the glomerular basement membrane (GBM), including laminin [71]; markers of tubular damage, such as N-acetyl-beta-D-glucosaminidase (NAG) and alfa 1 microglobulin [66, 71, 72].

Higher urinary concentrations of type IV collagen have been found in diabetics compared to controls, even in normoalbuminuric subjects [66, 70, 71, 73–83], suggesting that type IV collagen could be an early predictor of diabetic nephropathy. In an Asian multicenter study of nearly 700 diabetic patients, Tomino et al. showed that the urinary excretion of type IV collagen in diabetic patients increased gradually as renal diseases progressed [82].

Multiple studies have shown that urinary excretion of type IV collagen in type 2 diabetics relates to UAE [66, 71, 72, 74, 78, 79, 82–84]. In contrast, patients with nondiabetic chronic glomerulonephritis do not show this relationship [66].

Although type IV collagen excretion is higher in nondiabetic chronic kidney disease compared to healthy controls, type 2 diabetic patients with evidence of kidney disease have a significantly higher type IV collagen/albumin ratio compared to patients with nondiabetic nephropathy [66, 71, 74], suggesting that urinary type IV collagen can help to differentiate diabetic versus nondiabetic nephropathy.

In a prospective study, urinary type IV collagen was found to be more sensitive than albuminuria to detect renal damage in type 2 diabetic patients [72]. However, it has been reported that as many as 33% of microalbuminuric patients do not have increased urinary type IV collagen excretion [84]. In another study, Yagame et al. found that the area under the receiver operating characteristic (ROC) curve of albumin and type IV collagen was very similar, suggesting that UAE and urinary type IV collagen excretion have similar ability to detect early diabetic nephropathy [83].

In a follow-up study of 94 diabetic patients, Iijima et al. found that after 1 year, 25% of normoalbuminuric patients with increased urinary type IV collagen excretion developed microalbuminuria, and 75% stayed normoalbuminuric. The patients that stayed normoalbuminuric had a significant decrease in the urinary type IV collagen excretion, while the patients that developed microalbuminuria had a further increase in type IV collagen excretion [84].

Urinary type IV collagen excretion in type 2 diabetic patients is significantly associated with the duration of diabetes [71, 83]. Additionally, it is correlated with total serum cholesterol level [66] and inversely correlated with the reciprocal of serum creatinine [85]. However, it is not associated with diabetic retinopathy [71, 75]. Conflicting results have been published regarding the association of urinary type IV collagen excretion with blood pressure [71, 82, 84] and glycemic control [66, 71, 72, 74, 77, 79, 82–85].

Angiotensin-converting enzyme inhibitors (ACEIs) have been shown to decrease the type IV collagen urinary excretion in type 2 diabetic patients [86]. In contrast, low-dose ARBs failed to decrease the urinary type IV collagen excretion in a small nonrandomized, noncontrolled trial [87]. Further randomized, controlled studies are needed to conclude whether renin-angiotensin-aldosterone system blockade decreases the urinary excretion of type IV collagen.

5. Fibronectin

Fibronectin, a high-molecular-weight protein, is an intrinsic component of the glomerular extracellular matrix. It is produced in the liver, vascular endothelia, and platelets. Fibronectin is involved in coagulation, platelet function, and tissue repair. In diabetes it may reduce erythrocyte deformity and filterability [5].

Urinary fibronectin excretion is higher in diabetic patients compared to controls, but the difference is only significant for macroalbuminuric patients [88, 89]. In diabetics, urinary fibronectin excretion is higher in patients with microalbuminuria compared to normoalbuminuria [88]. Additionally, urinary fibronectin levels correlate with the progression of biopsy proven glomerular diffuse lesions [90].

The excretion of urinary fibronectin degradation products correlates with UAE [91], and urinary fibronectin excretion has a weak negative correlation with creatinine clearance, mostly in patients with overt proteinuria [88, 90].

Urinary fibronectin excretion might be a useful biomarker of diabetic nephropathy, but further studies are needed to determine its relevance compared to albuminuria.

6. Laminin

Laminin is a 900-kDa glycoprotein that is a normal component of basement membranes. It is considered that serum laminin cannot be filtered in the normal glomerulus, and the urinary laminin is derived from the kidneys [5]. It has been shown by immunohistochemistry that laminin is located in the mesangial expansion and thickened capillary basement membranes characteristic of diabetic nephropathy [92]. As expected, urinary laminin excretion correlates with the urinary excretion of type IV collagen, the main GBM constituent [71]. Because laminin is also found in the tubular basement membrane, it could be expected to find a relationship between urinary excretion of laminin and markers of tubular injury (i.e., NAG, alfa 1 microglobulin, beta 2 microglobulin, and kappa light chains), but conflicting results have been published regarding this correlation [71, 92, 93].

Urinary laminin excretion is higher in diabetic patients compared to healthy controls, even before the development of microalbuminuria [71, 75, 93]. However, there are conflicting results regarding the correlation of urinary laminin excretion with UAE [71, 92, 93].

Urinary laminin excretion increases with age, specifically in patients over 60 years of age [71, 92]. It is significantly correlated with the duration of diabetes [71], blood pressure [71], and glycemic control [71, 93].

Although urinary laminin excretion is higher in nondiabetic chronic nephropathy compared to controls, type 2 diabetic patients with evidence of nephropathy had significantly higher laminin/albumin ratio compared to patients with nondiabetic nephropathy [71], suggesting that urinary laminin excretion could help differentiate diabetic versus nondiabetic nephropathy.

Further studies are needed to determine the relevance of urinary laminin excretion in diabetic nephropathy.

7. Glycosaminoglycans

Glycosaminoglycans (GAGs), with molecular weight ranging between 13 and 30 kDa, are important components of the extracellular matrix, cellular membranes, and endothelial glycocalyx. GAGs are involved in regulation of cell proliferation and differentiation, cell-to-matrix binding, cell-to-cell interaction, and regulation of interleukin-1 production. They are also a major component of basement membranes [94]; heparan sulfate, the most prevalent glycosaminoglycan in the GBM, has been recognized as the main anionic component of the GBM [95, 96]. In diabetes, there is a decrease in heparan sulfate content in the mesangial matrix and GBM, resulting in an alteration of the charge-selectivity of the glomerular capillaries, which may in part contribute to the proteinuria that characterizes diabetic nephropathy [5]. Hyperglycemia reduces the synthesis of GAGs by the glomerular endothelial cells decreasing the heparan sulfate content of the glycocalyx and thus increasing the passage of albumin through the glomerular capillary wall without affecting the interendothelial junctions [97]. Additionally, the systemic endothelial glycocalyx damage coincides with the development of microalbuminuria [98].

GAGs are also present in the tubular basement membrane and a correlation between urinary markers of tubular damage (beta-2 microglobulin, NAG, and Tamm-Horsfall protein) and urinary GAGs excretion has been shown [99–101]. Ueta et al. report an association between urinary GAGs excretion and the severity of the GBM lesion in diabetics with good glycemic control. While in poorly controlled patients it is associated with the severity of the tubulointerstitial lesion [102].

Multiple studies have described an increased urinary GAGs excretion compared to controls, even in normoalbuminuric patients [94–96, 99, 100, 102–111]. But one study found similar urinary GAGs excretions in normoalbuminuric diabetic patients and healthy controls [102].

Discordant results have been published regarding the correlation of urinary GAGs excretion and UAE. Most of the studies found that GAGs excretion increases as albumin excretion increases [96, 99, 100, 108, 109], two studies did not find a correlation [95, 106], and one study found a decrease in GAGs excretion as UAE increases [101]. Torffvit et al. [101] found a decrease in sulphated GAGs excretion (but not of GAGs/creatinine ratio) between normoalbuminuric and albuminuric type 1 diabetic patients. In this study they used methods to identify sulphated GAGs, while other studies have used methods that do not react with sulphated groups. Diabetes is known to induce the synthesis of hyaluronan, a nonsulphated GAGs [112]. Thus, increased urinary excretion of degraded hyaluronan can explain the increase levels of GAGs obtained in previous studies [109]. It is important to standardize the methods to measure and report urinary GAGs to conclude whether there is a correlation between urinary GAGs excretion and UAE.

Diabetic patients with manifest nephropathy have increased urinary GAGs compared to patients with incipient nephropathy [94, 99, 108], and their sensitivity in patients with manifest nephropathy has been reported to be 100%, compared to 77% of albuminuria [99].

The prevalence of diabetic macroangiopathies in diabetic patients with elevated levels of urinary GAGs is significantly higher than in those with normal levels of urinary GAGs [100]. There is also a correlation between urinary GAGs excretion an diabetic neuropathy [99]. There are conflicting results regarding the correlation of urinary GAGs excretion and diabetic retinopathy; however, even the studies that reported a positive correlation show that the urinary GAGs excretion is not an independent risk factor for diabetic retinopathy [94, 99, 104, 110].

In diabetic patients, there is a correlation between urinary GAGs excretion and blood pressure [99, 101, 106, 108], but conflicting results have been published regarding their correlation with duration of diabetes [94, 96, 99, 106, 108, 110] and glycemic control [94, 96, 99, 101, 102, 106, 108, 109].

Urinary GAGs excretion could be a good marker for diabetic nephropathy and other complications of diabetes, but further studies and standardized methods of measurement of GAGs are needed before it is incorporated into clinical practice.

8. Immunoglobulin G

Immunoglobulin G (IgG) is a protein synthesized and secreted by plasma cells than is mainly involved in the secondary immune response. It is larger than albumin, with a molecular weight of 150 kDa and molecular radii of 62 Å, compared to albumin 65 kDa and 36 Å, respectively [113].

Total urinary IgG excretion is higher in diabetic patients compared to controls, even before they develop microalbuminuria [45, 46, 48, 114–116]. Urinary IgG excretion in normoalbuminuric diabetic patients predicts the development of microalbuminuria [56] and, unlike UAE, it correlates with the progression of glomerular diffuse lesions [117]. Intense glycemic control [115] and low-dose losartan [48] have been shown to revert the increased IgG excretion in these patients. Diurnal changes in systolic blood pressure significantly correlates with urinary IgG excretion, but not with UAE [64].

Urinary IgG excretion correlates well with urinary excretion of orosomucoid (a marker of inflammation and endothelial damage), transferrin and ceruloplasmin [114], but it has a weak and nonlinear relationship with UAE, indicating that the urinary excretion of IgG rises later and moves slower than that of albumin [50, 114].

Apart form using the total IgG urinary excretion as a marker of glomerular damage, the relationship between the urinary excretion of IgG and its isoform IgG4 has been used more specifically, as a marker of glomerular charge selectivity impairment. In general, the more anionic a protein is, the more difficult to pass through the glomerular barrier. Because IgG and IgG4 have similar size (strokes radius of 55 Å), but IgG4 is more anionic (isoelectric point of IgG 7.3 and IgG4 5.8) [19], the difference in their urinary excretion would be explained only by a charge, and not size, selectivity defect. The selectivity index (SI) is the tool that has been more widely used to assess the ratio between IgG and IgG4.

In microalbuminuric patients only IgG4 excretion is elevated, exemplified by a reduced SI in microalbuminuric compared to normoalbuminuric patients [16, 118]. While in macroalbuminuric patients excretion of both IgG and IgG4 are increased, shown by a similar IgG/IgG4 ratio but higher total IgG excretion in macroalbuminuric patients compared to microalbuminuric and normoalbuminuric patients [119, 120]. This suggests that the charge selectivity is lost in early diabetic nephropathy (microalbuminuric phase), which is difficult to evaluate in the macroalbuminuric phase because of the concomitant loss of size selectivity [14–16, 121–123].

The SI is not significantly different in normoalbuminuric diabetic patients compared to healthy controls [21, 39]; however, the urinary excretion of IgG4 and the SI has a significant correlation with UAE [17, 21, 39, 117, 124, 125]. Glycemic control [118], but not ACEI [125], increases the SI in type 1 diabetic patients with microalbuminuria. The clearance of IgG and IgG4 correlates with the duration of diabetes [15, 17].

The reduced SI in microalbuminuric patients does not correlate with markers of tubular injury (beta-2-microglobulin) [16], but a major disadvantage of measuring IgG and IgG4 is that both are reabsorbed in the tubules, causing the index to reflect the tubular and glomerular handling. Additionally, local production of IgG (e.g., prostate; seminal vesicles) and low-grade urinary tract infections can be other sources of error [21].

9. Ceruloplasmin

Ceruloplasmin, with a molecular weight of 151 kDa, is the major copper-carrying protein in the blood. It is more negatively charged than albumin [126] and therefore more difficult to be filtered by the glomerulus.

Urinary ceruloplasmin excretion is higher in type 2 diabetic patients compared to controls [114], even in the normoalbuminuric phase [48, 115]. It correlates well with albumin excretion rate [126, 127] and predicts the development of microalbuminuria in normoalbuminuric patients [56]. Glycemic control [115] and low-dose losartan [48] revert the increased urinary ceruloplasmin excretion in normoalbuminuric patients. And diurnal changes in the systolic blood pressure significantly correlate with urinary ceruloplasmin excretion, but not with UAE [64].

The ceruloplasmin/creatinine ratio is higher in diabetic nephropathy compared to nondiabetic nephropathy patients [128]. It has been reported that urine ceruloplasmin/creatinine ratio has a sensitivity of 90-91%, specificity of 61–66% and 75% concordance, in diagnosing diabetic nephropathy [127, 128].

Ceruloplasmin is a promising marker of diabetic nephropathy, but further studies are necessary to characterize its value compared to UAE, especially in type 1 diabetics, since all the studies have been done in type 2 diabetics.

10. Lipocalin-Type Prostaglandin D2 Synthase

Lipocalin-type prostaglandin D2 synthase (L-PGDS) is an enzyme-synthesizing prostaglandin D2 and a secretory protein of the lipocalin superfamily. It has similar chemical properties to albumin including anionic charge; however, it is much smaller in size (molecular weight 20–31 kDa), thus passing more easily through the glomerular capillary walls [129, 130]. L-PGDS is present in the peritubular interstitium and not in the tubular cells of nondiabetic patients while, in diabetic patients, it is present in the renal tubules [131].

Urinary L-PGDS excretion is higher in patients with any form of renal disease, except for males with IgA nephropathy, compared to controls. And has a sensitivity and specificity to diagnose renal disease of 67 and 86–93%, respectively [130]. It is more accurate than urinary type IV collagen, urinary markers of tubular injury (i.e., beta-2 microglobulin; NAG) and serum creatinine, but less accurate than UAE in diagnosing kidney disease [130].

Table 1: Overview of biomarkers of glomerular injury in diabetic nephropathy*.

Marker	DM1	DM2	Prior to MA**	Predicts MA***	UAE	Diabetic retinopathy	CV risk
Transferrin	+	+	+	+	+	+	$+^{\tau}$
IV-C	+	+	+		+	−	
Fibronectin	+	+			+		
Laminin	+	+	+		+/−		
GAGs	+	+	+		+/−	−	+
IgG	+	+	+	+	+		
Ceruloplasmin	−	+	+	+	+		
L-PGDS	−	+	+	+	+		+
IgM	+	+					+

DM1: studies in type 1 diabetic patients, DM2: studies in type 2 diabetic patients, MA: microalbuminuria, UAE: correlation with urine albumin excretion, CV: cardiovascular, IV-C: type IV collagen, GAGs: glycosaminoglycans, IgG: immunoglobulin G, L-PGDS: Lipocalin-type prostaglandin D2 synthase, IgM: immunoglobulin M. *: an empty space means not enough data published, **: presence prior to microalbuminuria, ***: predicts the development of microalbuminuria, "+/−": conflicting results are published, τ: transferrin is correlated with cardiovascular risk, but albumin is a better predictor of ischemic heart disease.

Because decreased glomerular filtration rate decreases L-PGDS urinary excretion, it is thought to be useful in early stages rather than advanced kidney disease [130].

Urinary L-PGDS excretion is higher in type 2 diabetic patients compared to controls, even in those without albuminuria [131, 132], and independently correlates with the urinary protein excretion [132]. Urinary L-PGDS excretion is useful in predicting future development of albuminuria (>30 mg/gCr) in normoalbuminuric patients with a sensitivity and specificity of 56–59% and 75–88%, respectively [130]. Overall, L-PGDS is more accurate than urinary type IV collagen, beta 2 microglobulin, NAG, and serum creatinine in predicting proteinuria [130]. Thus, urinary L-PGDS excretion is useful to detect early renal damage in normoalbuminuric patients. Combined with albumin, it increases the power to detect diabetic nephropathy in those patients already proteinuric [130]. Glycemic control decreases L-PGDS excretion to the normal range in normoalbuminuric patients [131].

In type 2 diabetic patients, the presence of higher L-PGDS excretion is independently associated with history of cardiovascular disease [133].

Urinary L-PGDS has been mainly studied in type 2 diabetic patients. It is a promising novel urinary marker of kidney disease, but further studies are needed to define its role in diagnosing diabetic nephropathy.

11. Immunoglobulin M

Immunoglobulin M (IgM), secreted by plasma cells, is the largest antibody in the human circulatory system. Due to its large molecular radius (120 Å), the appearance of IgM in the urine indicates an increased density of large, highly nonselective pores ("shunts") in the glomerular capillary wall, which implicates a severe size-selectivity defect [18, 116, 134, 135].

Increased urinary IgM excretion in patients with nondiabetic glomerular disease is associated with high degree of fibrosis and global glomerulosclerosis [134]. Furthermore, high urinary IgM excretion is a better predictor of decline in kidney function than albuminuria in these patients [134, 136, 137].

Increased urinary IgM excretion and IgG2/IgG4 ratio in macroalbuminuric type 2 diabetic patients compared to type 1 diabetic patients suggests that the proteinuria in type 2 diabetes is due to size-selectivity defects, while charge selectivity defects account for the proteinuria in type 1 diabetes [116].

Urinary IgM excretion is higher in macroalbuminuric type 2 diabetic patients compared to healthy controls but does not correlate with UAE or urinary alfa-1-microglobulin excretion (a marker of tubular injury) [116]. Increased urinary IgM excretion, independent of UAE, predicts cardiovascular mortality and progression to ESRD in diabetic patients [135, 138].

Urinary IgM excretion has not been studied as an early marker of diabetic nephropathy since it is associated with severe injury of the glomerular capillary wall. However, it is a promising marker that may predict the eventual need for renal replacement therapy and cardiovascular mortality. Urinary IgM has been mostly studied in small trials in the Nordic population and large trials in other ethnic groups are needed before it is implemented in clinical practice.

12. Conclusion

The current gold standard for detection and prediction of diabetic nephropathy and cardiovascular risk is albuminuria; however, it has several limitations. Makers that offer higher sensitivity and specificity for earlier detection of diabetic kidney disease and more accurate prediction of the progression to ESRD are needed. We reviewed nine biomarkers of glomerular injury implicated in diabetic kidney disease (Table 1).

Considering the results of the studies evaluating the biomarkers reviewed here, it is appealing to start utilizing them in clinical practice. However, the majority of publications reviewed are small cross-sectional studies, and there are

only a handful of longitudinal studies. Moreover, biomarkers only have clinical value if the results are reproducible, and none of the biomarkers reviewed here have been studied in more than 2 longitudinal trials. Hence, their clinical applicability needs to be confirmed in high-quality validation studies.

Furthermore, the majority of these studies, when reporting prediction of outcomes, use odds ratios or hazard ratios, which are inaccurate to predict the risk for individual subjects. Hellemons et al. in a systematic review of longitudinal trials, have suggested the use of area under the ROC curve, positive/true-positive fractions, net reclassification improvement, integrated discrimination improvement, or the discriminative likelihood ratio as better methods to validate new markers [4].

Another methodological issue is the use of "transition in albuminuria class" as an endpoint. For example, a patient who had an increase in UAE from 25 to 35 mg/day would be recognized as transitioning from normoalbuminuria to microalbumiruria, while a patient with increase from 35 to 295 mg/day, would not be considered as a progressor. We have to remind ourselves that the classification of albuminuria was based on the ability of dipstick to measure urinary albumin and not the association with disease. Furthermore, it is known that risk of cardiovascular events and renal morbidity is elevated in subjects in the "high normal" range of proteinuria [8, 31–33]. A more accurate way to report changes in albuminuria would be to assess the absolute changes in UAE.

Although many of the biomarkers reviewed here are promising, current data prevents us from making clear recommendations regarding their possible clinical use. Efforts on biomarker research should be directed at both new biomarker discovery and validation of published biomarkers on good quality, long-term, large longitudinal trials. Eventually, efforts should be made to develop a biomarker panel that is able to reliably assess diabetic nephropathy.

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