Judith Blaine *Editor*

Proteinuria: Basic Mechanisms, Pathophysiology and Clinical Relevance

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Introduction

Both albuminuria and proteinuria are sensitive markers of kidney disease and are strongly associated with kidney disease progression and increased risk of cardiovascular events. This volume will describe how albuminuria and proteinuria are measured in the clinical setting, the prognostic implications of increased urinary albumin or protein excretion, and the pathophysiology underlying the development of proteinuria. In addition, diseases or patterns of disease that commonly result in albuminuria or proteinuria will be described as well as the most recent developments in understanding the basic mechanisms underlying these diseases and how these findings have been translated into therapies.

While new bench techniques have significantly increased our understanding of how the kidney handles serum proteins, therapeutic options to treat proteinuria are limited, and there is still much progress to be made in developing targeted and effective agents to treat proteinuric renal diseases.

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Chapter 1
Evaluation and Epidemiology of Proteinuria

Judith Blaine

Abbreviations

AASK	African-American Study of Kidney Disease and Hypertension
ACE-I	Angiotensin converting enzyme inhibitor
AKI	Acute kidney injury
ARB	Angiotensin receptor blocker
CRIC	Chronic Renal Insufficiency Cohort
eGFR	Estimated glomerular filtration rate
ERAs	Endothelin receptor antagonists
ESRD	End stage renal disease
FSGS	Focal segmental glomerulosclerosis
MDRD	Modification of Diet in Renal Disease
NHANES	National Health and Nutrition Examination Survey
RAA	Renin angiotensin aldosterone system
RAS	Renin angiotensin system
REIN	Ramipril Efficacy in Nephropathy
UACR	Urine albumin-to-creatinine ratio
UPCR	Urine protein-to-creatinine ratio

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1.1 Measurement of Proteinuria

Normal urinary protein excretion is defined as urine protein excretion of less than 150 mg/day or urinary albumin excretion of less than 30 mg/day although increasing evidence from epidemiological studies suggests that there are increased risks of renal disease progression and cardiovascular morbidity and mortality well below this threshold (see below) [1–3]. In normal individuals, approximately 20 % of the total urinary protein excreted per day is albumin with the remainder consisting of low molecular weight proteins, Tamm-Horsfall proteins and immunoglobulin fragments.

There are a number of methods commonly used to measure protein excretion in the urine: urine dipstick, spot urine protein to creatinine ratio and a 24 h urine collection [4]. The urine dipstick detects primarily albumin and is much less sensitive at detecting other urinary proteins such as immunoglobulins. In addition, the dipstick is semi-quantitative (0 to 4+) and the results are very dependent on urinary concentration. While precise quantitation is not possible when using the dipstick, 1+ on urinary dipstick corresponds to approximately 30 mg of protein per dl; 2+ corresponds to 100 mg/dl, 3+ to 300 mg/dl, and 4+ to 1,000 mg/dl [5]. In one study the likelihood of excreting a gram or more of protein a day (as measured by the urine protein-to-creatinine ratio) was 7 % when urine dipstick protein value was 1+ or 2+, 62 % when dipstick protein value was 3+, and 92 % when dipstick protein value was 4+ [6]. False positive results may also occur with gross hematuria (urocrit > 1 %) [7], a highly alkaline urine which may indicate bacterial contamination [8] or the use of certain antiseptic wipes such as those containing chlorhexidine for obtaining clean catch samples [8]. The dipstick is also insensitive to albumin concentrations below 10–20 mg/dl.

Quantitative methods to assess urinary protein excretion include the spot urine protein-to-creatinine ratio (UPCR) and a 24 h urine collection. The UPCR is measured on a random urine sample, preferably an early morning sample, and is calculated by taking the ratio of the urinary protein to the urinary creatinine (assuming the same units (mg/dl) for each) [9]. The resulting ratio is taken to be the urinary protein excretion in grams per day [10]. For example, a random urine sample with a spot urine protein of 100 mg/dl and a spot urine creatinine of 50 mg/dl would indicate excretion of 2 g urinary protein a day. An underlying assumption in using the UPCR to estimate daily protein excretion in the urine is that the amount of creatinine excreted in the urine by the individual is 1 g/day. This is not necessarily true as men excrete more creatinine than women due to greater muscle mass and, after the age of 50, urinary creatinine excretion declines due to progressive loss of muscle mass. A measure of daily urinary albumin excretion can be estimated by calculating the urinary albumin-to-creatinine ratio (UACR) obtained by dividing the amount of albumin measured in a random urine sample by the amount of creatinine. The advantage of the UPCR or UACR compared to a 24 h urine protein collection is the ease of collection. A urine sample can often be obtained at an office visit allowing more rapid evaluation of whether a particular treatment designed to lower proteinuria is efficacious.

A 24 h urine collection has long been considered the gold standard for measuring proteinuria. A concomitant urine creatinine should also be obtained with the 24 h urinary protein measurement to evaluate the adequacy of collection. Men under the age of 50 should excrete 20–25 mg/kg lean body weight urinary creatinine per day and women under the age of 50 should excrete 15–20 mg/kg lean body weight creatinine. Thus, a healthy adult male with a lean body mass of 70 kg should excrete 1400–1750 mg creatinine per day. In a healthy adult male, a 24 h urinary creatinine excretion much less than 1400 mg or much greater than 1750 mg would indicate an under or over collection. While considered the gold standard, a 24 h urinary protein collection is often cumbersome to collect. Several studies have found reasonable correlation between an estimation of urinary protein excretion as measured by a 24 h urine collection compared to the UPCR in both the general population and kidney transplant recipients at lower levels of urinary protein excretion (<6 g/day) [10–13].

1.2 Epidemiology

An accurate assessment of how many individuals in the United States are proteinuric is difficult as proteinuria can be transient (especially at levels <1 g/day, see below) and differences in the methods used to measure proteinuria can yield different results. Nonetheless, data from the National Health and Nutrition Examination Survey (NHANES) 1999–2004 survey indicate that 8.1 % of participants had at least one albuminuria measurement of >30 mg/g [14].

Numerous studies have shown that proteinuria or albuminuria is strongly correlated with increased risk of progression of kidney disease [1–3, 15, 16]. In a meta-analysis of nine general population cohorts with 845,125 participants and an additional eight cohorts with 173,892 patients without chronic kidney disease, adjusted hazard ratios for progression to end stage renal disease (ESRD) at albumin-to-creatinine ratios of 30, 300, and 1000 mg/g were 5, 13, and 28, respectively, compared to individuals with albumin-to-creatinine ratio of 5 mg/g [1]. It is important to note that the risk of ESRD was increased even in those with an ACR of 30 mg/g which is currently considered close to normal. In another study of 107,192 Japanese individuals, proteinuria was the most powerful predictor of ESRD risk over 10 years [17]. In the 274 patients in the Ramipril Efficacy in Nephropathy (REIN) trial, urinary protein excretion was the only baseline variable that correlated with loss of estimated glomerular filtration rate (eGFR) and progression to ESRD [18]. Similarly, in the Modification of Diet in Renal Disease (MDRD) study higher proteinuria at baseline was associated with more rapid loss of GFR [19] and in the African-American Study of Kidney Disease and Hypertension (AASK) trial, for each twofold increase in proteinuria a mean ± SE 0.54 ± 0.05-ml/min per 1.73 m^2 per year faster GFR decline was seen [20].

Increased urinary protein excretion is associated with increased risk of cardiovascular morbidity and mortality in both the general population [3] and those at high risk of cardiovascular events [2]. In a Canadian study of 920,985 adults, mortality of

individuals with heavy proteinuria and eGFR > 60 ml/min/1.73 m^2 was more than twofold higher than that for those with eGFR < 45 ml/min/1.73 m^2 and no proteinuria at baseline [3]. The mortality findings are also independent of traditional cardiovascular risk factors such as diabetes. In a study of 1,024,977 participants (128,505 with diabetes), the hazard ratio of mortality outcomes for ACR 30 mg/g (vs 5 mg/g) was 1.50 (95 % confidence interval 1.35–1.65) for those with diabetes vs 1.52 (1.38–1.67) for those without [21]. Similarly, in the 3939 patients enrolled in the Chronic Renal Insufficiency Cohort (CRIC), proteinuria and albuminuria were better predictors of stroke risk than eGFR [22]. Meta analyses have shown that albuminuria >300 mg/day or proteinuria are associated with a 1.5–2.5-fold increased risk of cardiovascular mortality [23, 24].

Proteinuria or albuminuria is also associated with an increased risk of developing hypertension or acute kidney injury (AKI). In the 9,593 patients in the Atherosclerosis Risk in Communities study, elevated albuminuria consistently associated with incident hypertension [16]. In 8 general-population cohorts (total of 1,285,049 participants) and 5 chronic kidney disease (CKD) cohorts (79,519 participants), increased albuminuria was strongly associated with AKI as evidenced by the fact that the risk of AKI at ACR of 300 mg/g was 2.73 (95 % CI, 2.18–3.43) compared with ACR of 5 mg/g [25].

1.3 Evaluation of the Individual with Proteinuria

An individual identified as having albuminuria or proteinuria should have an examination of the urinary sediment for any evidence of hematuria or red cell casts that could indicate the presence of a nephritic glomerulonephritis. In addition, kidney function should be assessed and the proteinuria should be quantified using a spot urine protein-to-creatinine ratio (UPCR) measurement or a 24 h urine collection. If possible the spot UPCR should be correlated with a 24 h urine protein collection as the 24 h collection is considered to be the gold standard. In those with normal kidney function and a bland urine sediment, a determination should be made as to whether the proteinuria is transient or whether the individual has orthostatic proteinuria. Transient proteinuria, which is often <1 g/day, occurs when a repeat test for albuminuria or proteinuria is negative. Transient proteinuria is common in children, occurring in up 5 % to 15 % of school-aged children [26, 27]. If a repeat measurement of albuminuria or proteinuria is negative, no further workup is needed [26].

Orthostatic proteinuria is also common in those under the age of 30 [28]. Orthostatic proteinuria is diagnosed by the finding of proteinuria in a urine sample collected after the patient has been upright for several hours and no proteinuria in a sample collected immediately after an individual has been supine for several hours. When quantified, orthostatic proteinuria is usually <1 g/day and the condition is not associated with any long term adverse renal outcomes [26, 28].

Persistent proteinuria can result from a number of causes (Table 1.1) and generally warrants referral to a nephrologist especially when the proteinuria is nephrotic

Table 1.1 Causes of proteinuria

	Persistent proteinuria	
Transient proteinuria	Renal cause	Non-renal cause
Exercise	Glomerulonephritis	Nephrolithiasis
Fever	Diabetes	Urinary tract infections
Albumin infusion	Medications	Genito-urinary malignancies
	Inflammatory diseases	
	Infection	
	Malignancies	
	Infiltrative diseases	
	Hypertension	
	Acute interstitial nephritis	
	Heavy metal intoxication	

(>3.5 g/day). As long as there are no contraindications to biopsy, a kidney biopsy is generally performed in those with nephrotic range proteinuria or those in whom proteinuria steadily increases with serial measurements or in individuals with an active urinary sediment (hematuria or cellular casts). Kidney biopsy may not be performed in individuals who are highly likely to have diabetic nephropathy or in those with proteinuria consistently <1 g/day and in whom a kidney biopsy is unlikely to change management.

1.4 Treatment

RAAS Blockade Besides therapies aimed directly at treating the underlying cause of proteinuria which may include immunosuppressive medications for diseases such as focal segmental glomerulosclerosis, membranous nephropathy or lupus, a mainstay of treatment is lowering of intraglomerular pressure through the use of angiotensin converting enzyme inhibitors (ACE-I) or angiotensin receptor blockers (ARBs). The dose of ACE-I or ARB should be maximized as tolerated by blood pressure and renal function as studies have shown that greater decrements in proteinuria are associated with better renal outcomes in both diabetic and nondiabetic patients. In a trial of 40 type I diabetics treated with enalapril versus other non ACE/ARB antihypertensives, the enalapril group had a more than 50 % reduction in loss of eGFR compared to the non ACE/ARB group over 2.2 years of follow up [29]. Lewis et al. showed in a trial of 409 patients with insulin-dependent diabetes that treatment with captopril versus placebo resulted in a highly significant decrease in the number of subjects who had a doubling of their baseline serum creatinine at the end of 4 years, despite similar blood pressure control in the 2 groups [30]. In the Lewis trial, treatment with captopril also resulted in a 50 % reduction in the combined end point of need for dialysis, transplantation or death [30]. Several post-hoc analyses of trials including diabetic patients have shown a similar beneficial effect

of ACE-I or ARB on reduction in proteinuria and slowing of loss of GFR as well as a significant decrease in cardiovascular events [31].

The first trial to demonstrate the benefit of renin-angiotensin inhibition in proteinuric nondiabetic patients was the REIN trial which examined renal outcomes in nondiabetic patients divided into tertiles based on baseline proteinuria (0.5–1.9 g, 2.0–3.8 g or >3.8 g/day). Subjects were randomly assigned to receive either ramipril (an ACE-I) or non ACE/ARB antihypertensive therapy to achieve a diastolic blood pressure ≤90 mmHg. Despite equivalent blood pressure control in both groups, treatment with ramipril resulted in a greater reduction in proteinuria than in the non ACE/ARB group and this decrease translated into a 50 % decrease in progression to ESRD over 42 months of follow up [32]. Post-hoc analysis of other trials examining proteinuria reduction have shown similar benefit in other nondiabetic populations with baseline proteinuria >1 g/day [31].

While ACE or ARB monotherapy should be maximized as tolerated, these agents should not be combined as trials such as ONTARGET have shown that a combination of ACE-I and ARB leads to increased adverse events (hypotension, syncope and renal dysfunction) without any increased benefit [33]. While dual ACE/ARB therapy was shown to result in a greater reduction in proteinuria than monotherapy in the ONTARGET trial, patients in the dual therapy group had a significant increase in the primary renal outcome (doubling of serum creatinine, dialysis or death) as well as the secondary renal outcome (doubling of serum creatinine or dialysis) [34]. Although ACE and ARB therapy have been considered to be equivalent in efficacy in decreasing proteinuria, a recent meta analysis of trials using ACE-I or ARB in diabetic patients demonstrated that ACE-I reduced all-cause mortality, CV mortality, and major CV events in patients whereas ARBs had no beneficial effects on these outcomes [35].

Endothelin Receptor Antagonists (ERAs) Renal endothelin modulates sodium and water handling, renal vasoconstriction, acid/base handling and podocyte function [36]. Infusion of endothelin-1 (ET-1) into rats results in podocyte foot process effacement and proteinuria [37] and endothelin-1 also plays a role in cellular proliferation and fibrosis. Renal production of endothelin-1 is increased in diabetic nephropathy, hypertension and experimental models of focal segmental glomerulosclerosis (FSGS) and ET-1 levels are increased in individuals with chronic kidney disease [36]. While a few trials using ERAs in diabetic nephropathy have shown modest reductions in urinary albumin excretion, use of these agents has been limited by fluid retention and adverse events at higher doses. The ASCEND trial randomized 1392 patients with type 2 diabetes already on RAS blockade to the ERA avosentan versus placebo. The median eGFR of individuals in the trial was ~ 33 ml/min/1.73 m^2 and the median albumin-to-creatinine ratio (ACR) was 1500 mg/g [38]. While patients in the avosentan group had a reduction in albuminuria at 4 months, the trial was stopped prematurely due to adverse cardiovascular events in the avosentan group including a threefold increased risk of congestive heart failure. Subsequent trials have used lower doses of ERAs and excluded patients with a history of heart failure. These studies have shown significant reductions in the ACR in

individuals already on maximal RAS blockade who received ERAs. In general ERAs at a dose of ≤1.25 mg/d were well tolerated [36]. There are currently 2 trials using endothelin receptor antagonists in development—one examining the use of ERAs in type 2 diabetic patients on maximally tolerated RAS blockade and the other examining ERA use in patients with FSGS. (See https://clinicaltrials.gov/ct2/results?term=endothelin+receptor&Search=Search for more details).

1.5 Summary

Proteinuria or albuminuria is a marker of kidney damage and strongly associated with progression of kidney disease and cardiovascular mortality. Initial evaluation of the proteinuric patient involves quantitation of proteinuria and evaluation of whether proteinuria is the result of renal or extra-renal pathology. First line treatment of proteinuria involves the use of ACE-I or ARB. A better understanding of the mechanisms underlying the development of proteinuria will ultimately result in new therapies to decrease urinary protein excretion and slow kidney disease progression.

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Chapter 2
Glomerular Mechanisms of Proteinuria

Evgenia Dobrinskikh and Judith Blaine

Abbreviations

ACTN4	Actinin alpha 4
Angpt	Angiopoietin
APOL1	Apolipoprotein L1
COX2	Cyclooxygenase 2
CXCL12	1/C-X-C chemokine ligand 12
CXCR4	C-X-C chemokine receptor 4
ESRD	End stage renal disease
FSGS	Focal segmental glomerulosclerosis
GAGs	Glycosaminoglycans
GBM	Glomerular basement membrane
GEC	Glomerular endothelial cells
GFB	Glomerular filtration barrier
Grb2	Growth-factor receptor binder 2
GSC	Glomerular sieving coefficient
GTP	Guanosine-5′-triphosphate
IgG	Immunoglobulin
L	Liter
LAMB2	Lamininβ2
NcK	Non catalytic kinase
nm	Nanometer
NPHS1	Gene that encodes nephrin
NPHS2	Gene that encodes podocin
N-WASP	Wiskott–Aldrich syndrome protein

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PEC	Parietal epithelial cells
PI3k	p85/phosphatidylinositol 3-kinase
PLCg	Phospholipase C gamma
SH2/3	Src homology 2 (SH2)/Src homology 3 (SH3)
TAK1	Transforming growth factor (TGF)-β activated kinase 1
Tie	Tyrosine-protein kinase receptor
TRPC6	Transient receptor potential cation channel subfamily 6
VEGFA	Vascular endothelial growth factor a
VEGFR	VEGF receptor
WT1	Wilms tumor protein 1
ZO-1	Zonula occludens-1

2.1 Introduction

The normal kidney filters 180 L of plasma a day and yet the final 1–2 L of urine produced per day contains almost no serum proteins. The glomerular filtration barrier (GFB) plays an important role in preventing the passage of serum proteins into the ultrafiltrate. While the precise mechanisms involved in limiting the passage of serum proteins into the final urine remain to be fully determined, recent genetic and advanced imaging methods have significantly furthered our understanding of the role of the GFB in this process.

2.2 Structure of the Glomerulus

Each glomerulus is made up of an afferent arteriole which gives rise to a tortuous filtration unit, the glomerular tuft, which finally leads to the efferent arteriole. Four distinct cell types are found within the glomerular tuft: glomerular endothelial cells (GECs), podocytes (also known as visceral epithelial cells), mesangial cells, and parietal epithelial cells (PECs) which line Bowman's capsule [1] (Fig. 2.1). The glomerular filtration barrier, consisting of fenestrated GECs, the glomerular basement membrane (GBM) and podocytes, forms the primary barrier to filtration of serum proteins such as albumin and immunoglobulin (IgG) into the ultrafiltrate.

Glomerular Endothelial Cells GECs within the GFB are distinctive in that they lack surrounding smooth muscle cells and contain pores that are 60–100 nm wide [2]. Theoretically these pores are wide enough to accommodate albumin which has a radius of 3.5 nm but GECs are also covered in a negatively charged glycocalyx that reduces the effective size of the endothelial pores [3]. The glycocalyx, consisting of proteoglycans bound to polysaccharide chains called glycosaminoglycans (GAGs), glycoproteins, and glycolipids is also believed to provide an important scaffold for signaling molecules as well as to sense mechanical stress [3]. Evidence

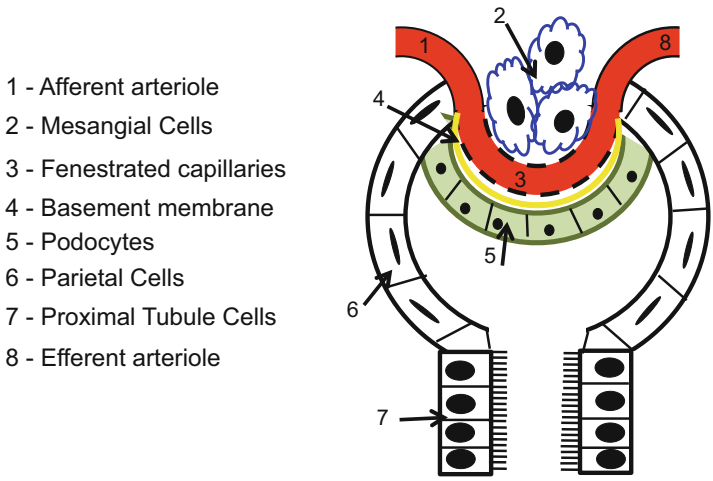

Fig. 2.1 Schematic diagram of a glomerulus. The glomerular filtration barrier is made up of fenestrated endothelial cells, the glomerular basement membrane and podocytes

for the role of the glomerular endothelial cell and the associated glycocalyx in glomerular albumin filtration comes from studies demonstrating that enzymatic destruction of the endothelial glycocalyx increases albuminuria and results in alterations in glomerular size and charge selectivity [4, 5]. In addition, aging and diabetes have been shown to damage the endothelial glycocalyx resulting in increased albuminuria [6].

Glomerular Basement Membrane The glomerular basement membrane (GBM) is another key component of the GFB. The GBM is a thin (250–400 nm) layer formed by fusion of the basement membranes of glomerular endothelial cells and podocytes [7]. Type IV collagen makes up ~50 % of the GBM [8]. Other predominant GBM components include laminins, nidogen, and heparan sulfate [9]. Mutations in laminin or collagen IV lead to severe filtration defects and progressive renal disease in humans indicating that these proteins are particularly important for the structure and function of the GBM [10, 11]. The GBM also stabilizes the glomerular filtration barrier by providing a scaffold for endothelial cell and podocyte attachment. High-resolution microscopy techniques have revealed that the GBM contains a network of fibrils ranging from 4 to 10 nm in diameter and that structural components such as laminin and collagen IV are precisely arranged. Podocyte foot processes attach to the GBM via vinculin, talin and integrins which bind to GBM collagen IV and laminin [12].

Podocytes The final barrier to protein filtration within the glomerular tuft is the podocyte. It has long been known that podocyte loss correlates with the severity of proteinuria in both humans and animals and that flattening or effacement of podocyte foot processes also leads to marked increases in albuminuria [13–16]. Since

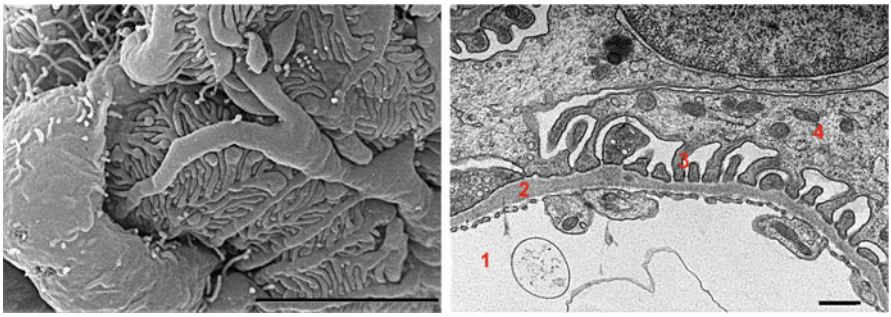

Fig. 2.2 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of a podocyte. *Left panel*: SEM of a podocyte. Note the large cell body which gives rise to several processes. Scale bar: 500 nm. *Right panel:* TEM of a podocyte. *1*, endothelial cell; *2*, glomerular basement membrane; *3*, podocyte foot process; *4*, major process. Scale bar: 1 μm. Images courtesy of Patricia Zerfas, Division of Veterinary Resources, Office of Research Services, National Institutes of Health

podocytes are highly specialized and terminally differentiated cells, their loss cannot be easily compensated and a progressive decrease in podocyte number leads to progressively increasing proteinuria. Recent evidence also demonstrates that podocytes play an active role in handling serum proteins such as albumin and IgG (see below).

2.3 Podocyte Structure and Function

Since podocytes are believed to form the primary barrier within the glomerulus to filtration of serum proteins into the urine, podocyte structure and function will be discussed in detail below as will genetic mutations in podocyte proteins that give rise to proteinuria.

Podocyte Structure Podocyte structure is integral to podocyte function. Podocytes have a unique morphology—a large cell body gives rise to multiple processes that split into larger major processes and smaller processes known as foot processes [17] (Fig. 2.2). The predominant structural components of the large processes are microtubules whereas actin is the main structural element in the foot processes. Podocytes tightly encircle the glomerular capillaries and foot processes from adjacent podocytes are connected to each other via a structure known as the slit diaphragm [18]. The slit diaphragm, which is a modified adherens junction, contains several proteins that play an important role in signaling and maintenance of the filtration barrier. Nephrin and neph1, which localize to the slit diaphragm, are members of the IgG superfamily and play an important role in signaling and glomerular permeability [19]. Phosphorylation of tyrosines within the cytoplasmic tails of nephrin and neph1 by the kinase Fyn allows recruitment of Src homology 2 (SH2)/Src homology 3

(SH3) adaptor proteins, including non catalytic kinase (Nck)1/2, growth-factor receptor binder 2 (Grb2), p85/phosphatidylinositol 3-kinase (PI3K), and phospholipase C gamma (PLCγ) [20]. This in turn leads to alterations in actin dynamics mediated through the actin nucleation protein neuronal Wiskott–Aldrich syndrome protein (N-WASP) [21]. Actin dynamics in podocytes are also regulated by the Rho-family of small GTPases—RhoA, Rac1 and Cdc42 [22]. Regulation of actin dynamics within podocyte foot processes is a highly complicated process and is currently the focus of intense investigations.

Since the slit diaphragm forms a junction between adjacent podocytes, it is not surprising that this structure also contains proteins found in other adherent and tight junctions such as zonula occludens-1 (ZO-1), p-cadherin, spectrins, catenins and occludins [1, 23]. ZO-1 binds to neph1 and disruption of this interaction leads to proteinuria in mice [24].

Podocyte Protein Handling Under Normal Conditions Increasing evidence suggests that podocytes play an active role in handling serum proteins such as albumin and IgG under normal conditions. Cultured podocytes have been shown to take up albumin [25, 26]. While the receptors involved in albumin and IgG uptake in podocytes have not been definitively identified, studies have shown that the receptor involved in albumin uptake is inhibited by statins [25]. Furthermore, podocyte albumin endocytosis is caveolin-1-dependent as inhibition of caveolin-1 leads to a significant reduction in albumin uptake in cultured human podocytes [26]. In vitro studies have shown that endocytosed albumin is both degraded and transcytosed with ~20 % of the endocytosed albumin routed to the lysosome for degradation and ~80 % transcytosed [26, 27].

Albumin endocytosis, degradation and transcytosis have been shown to occur in podocytes in vivo using multiphoton intravital microscopy, a dynamic imaging technique that allows for examination of protein trafficking in intact podocytes in real time. The amount of albumin shown to be filtered by the podocyte varies widely and is a matter of active investigation. Podocyte albumin filtration is measured by a value known as the glomerular sieving coefficient (GSC). Using intravital multiphoton microscopy, GSC values have been found to range from a low value of ~0.002 (which would be equivalent to ~14 g albumin filtered per day in humans) [28] to a high GSC value of ~0.035 (equivalent to ~250 g albumin filtered per day) [29]. The GSC value and thus the amount of albumin filtered has also been shown to differ based on the strain of rodent used and factors such as temperature [30]. A recent intravital study has also demonstrated that albumin vesicles in podocytes in vivo are routed to the lysosome or transcytosed, in accord with previous studies in cultured podocytes [31].

Albumin modification via lipidation or glycation is also thought to alter protein trafficking in podocytes. Shaw et al. have shown that albumin lipidation increases podocyte macropinocytosis via a pathway involving free fatty acid receptors [32].

Podocyte Production of Autocrine and Paracrine Factors Podocytes produce a number of factors required for the correct development and function of the glomerular filtration barrier. Vascular endothelial growth factor a (VEGFA) produced by podocytes

plays a key role in glomerular development. Inducible deletion of podocyte VEGFA in diabetic mice results in glomerular endothelial cell damage and progression of diabetic nephropathy [33]. Deletion of deletion of transforming growth factor (TGF)-β activated kinase 1 (TAK1) from podocytes results in delayed glomerulogenesis and abnormal glomerular capillary formation [34].

VEGF signals via binding to VEGF receptors. Deletion of the soluble form of the VEGF receptor VEGFR1 (also known as sFlt1) in podocytes results in massive proteinuria and renal failure. sFlt1 produced by podocytes signals in an autocrine fashion by binding to glycosphingolipids in the cell membrane, initiating a signaling cascade that ultimately results in actin cytoskeleton rearrangement [35].

Another signaling system involved in podocyte/endothelial cell crosstalk is the angiopoietin/Tie-2 system. Podocytes produce angiopoietin (Angpt) 1 and 2, which bind to the tyrosine-protein kinase (Tie2) receptor. Deletion of Angpt1 during murine embryonic development leads to abnormalities in glomerular capillaries and disruption of the glomerular basement membrane [36]. Overexpression of Angpt2 in podocytes leads to apoptosis of glomerular endothelial cells and increased albuminuria. Taken together, these results suggest that a balance in Angpt1/Angpt2 signaling is important for maintaining the integrity of the GFB [37].

Stromal cell-derived factor 1/C-X-C chemokine ligand 12 (CXCL12) is another factor involved in podocyte/endothelial cell crosstalk. Podocytes produce CXCL12 which acts on the C-X-C chemokine receptor 4 expressed by endothelial cells (CXCR4). Both CXCL12 and CXCR4 knockout mice have abnormal blood vessel formation with ballooning of the glomerular capillaries [38].

Podocytes not only produce factors required for endothelial cell development but also secrete factors required for formation of the glomerular basement membrane. Podocytes secrete α3, α4 and α5 collagen chains that are the key components of type IV collagen, a major component of the GBM [39]. In addition podocytes produce laminin-1 and 11 chains that are also necessary for GBM formation [40].

Genetic Mutations Mutations in proteins expressed in podocytes cause proteinuria. Kestila et al. were the first to demonstrate that mutations in NPHS1, the gene that encodes nephrin, led to development of congenital nephrotic syndrome of the Finnish type [41]. Subsequently, mutations in at least 45 other genes, the vast majority of which are important for podocyte structure or function, have been identified as causative for various forms of nephrotic syndrome in humans [42]. Genetic mutations are much more likely to be a cause of nephrotic syndrome in children than in adults. In children, genetic abnormalities account for 12–22 % of patients with nephrotic syndrome [43]. The most common genetic mutations resulting in nephrotic syndrome in children are found in 4 genes: NPHS1 (encodes nephrin), NPHS2 (encodes podocin) [44], WT1 (encodes a podocyte nuclear transcription factor) [45], and LAMB2 (encodes lamininβ2) [46]. While only a small fraction of nephrotic syndrome diagnosed in adulthood is due to genetic mutations, mutations in the following genes are among those more commonly associated with nephrotic syndrome: INF2 (encodes a member of the diaphanous inverted formin family) [47], TRPC6 (encodes a cationic channel that preferentially passes calcium) [48], and ACTN4

(encodes a member of the spectrin family that bundles actin) [49]. In general, proteinuria due to nephrotic syndrome is poorly responsive to immunosuppressive treatment.

African Americans have a three to fourfold increased risk of end stage renal disease (ESRD) and a 7–8 fold increased risk of focal segmental glomerulosclerosis (FSGS, a hall mark of podocyte damage). Mutations in APOL1, the gene encoding apolipoprotein L1, account for all of this increased risk [50, 51]. There are 2 common types of mutations in APOL1 known as the G1 and G2 variants. Increased risk is conferred when an individual has two APOL1 risk variants (G1/G1, G1/G2 or G2/G2) [52]. While ApoL1 is expressed in podocytes (as well as portions of the renal vasculature and proximal tubules) [53], the mechanisms whereby mutations in APOL1 increase the risk of FSGS and ESRD in African Americans remain unknown. The mechanism, however, is thought to be intrinsic to the kidney as kidneys heterozygous for APOL1 mutations transplanted into patients that are homozygous for APOL1 survive as long as comparable transplants, whereas kidneys with 2 APOL1 mutations fail at higher rates than those with zero or one mutation [54–56].

Deleterious Effects of Albumin on Podocytes Proteinuria is strongly and independently correlated with kidney disease progression and higher levels of proteinuria are associated with increased risk of kidney failure [57–59]. While the mechanisms involved in determining how proteinuria might lead to kidney failure remain to be determined, it has been shown that heavy proteinuria can result in protein inclusion droplets in podocytes [60–62]. In addition, several studies using cultured podocytes and in vivo models have shown that albumin exposure upregulates production of pro-inflammatory cytokines and increases podocyte apoptosis [62–64]. Since podocytes are terminally differentiated cells with limited regenerative capacity, death of sufficient numbers of podocytes leads to glomerulosclerosis and renal failure. Albumin exposure in cultured podocytes also upregulates endoplasmic reticulum stress and causes podocyte cytoskeleton rearrangement [65, 66]. In addition, Agrawal et al. have shown both in vivo and in vitro that albumin exposure induces podocyte production of cyclooxygenase 2 (COX-2), a key player in upregulating the inflammatory response [67].

2.4 Summary

While all three components of the glomerular filtration barrier, endothelial cells, the glomerular basement membrane and podocytes, contribute to glomerular permselectivity, the final barrier to serum protein filtration is formed by podocytes and the podocyte slit diaphragm. The importance of the podocyte in maintaining the GFB is underscored by genetic mutations in podocyte proteins that result in heavy proteinuria. The mechanisms involved in albumin and IgG trafficking in podocytes are an area of active investigation and recent advances in high resolution imaging techniques have enabled examination of these processes in living podocytes in real time.

Since albumin accumulation within podocytes is thought to contribute to podocyte death and glomerulosclerosis, a mechanistic understanding of the role podocytes play in protein handling across the filtration barrier may ultimately lead to attenuation of proteinuria and slowing of kidney disease progression.

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Chapter 3
Tubular Mechanisms in Proteinuria

Sudhanshu K. Verma and Bruce A. Molitoris

Abbreviations

AKI	Acute kidney injury
AP1	Activator protein 1
BAD	Bcl-2 associated death promoter
BASP	Brain abundant signal protein 1
Bcl-2	B cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BMP	Bone morphogenic protein
DAMP	Danger-associated molecular patterns
DT	Diphtheria toxin
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal related kinases
FADD	Fas associated protein with death domain
FcRn	Neonatal Fc receptor
FITC	Fluorescein isothiocyanate
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
IgG	Immunoglobulin
IL	Inter-leukin
K_d	Dissociation constant
kD	Kilo dalton
MAP	Mitogen activated protein
MCP	Monocyte chemoattractant protein

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MHC	Major histocompatibility complex
MWF	Munich-Wistar Fromter
NF-kB	Nuclear factor kappa-light chain-enhancer of activated B cells
NHE3	Na^+/H^+ exchanger isoform3
NLR	Nod like Receptor
NLRP3	NOD- like receptor family Pyrin domain containing 3
OK	Opossum kidney
PDGF	Platelet derived growth factor
PKB	Protein kinase B
PPAR	Peroxisome proliferator activated receptor
PT	Proximal tubule
PTC	Proximal tubular cell
RANTES	Regulated on activation normal T cell expressed and secreted
RAP	Receptor associated protein
RCT	Random control trial
TGF	Tumor growth factor
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptors
UTP	Uridine Tri-phosphate
α SMA	α-Smooth muscle actin

3.1 Introduction

The kidneys are responsible for maintaining the homeostasis of body fluids by the regulation of water, electrolyte and acid base balance, and the excretion of uremic toxins. In the last two decades another important function of kidney has emerged—its role in protein metabolism. Chronic progressive nephropathies, independent of the type of initial insult, are characterized with consistently high levels of proteinuria. Proteinuria can occur in various forms and different levels of severity. Based on the amount of protein in the urine proteinuria is classified as nephrotic or non-nephrotic. Depending upon the underlying pathological damage it can be either glomerular or tubular. In either glomerular or tubular proteinuria, the proximal tubular cell (PTC) plays fundamental, physiologic, synergistic, interactive, and dynamic roles in the renal handling of proteins. Therefore, the goal of this chapter is to review the role, mechanism and pathways of tubular reabsorption of protein along the nephron primarily by proximal tubular cells under normal and pathological conditions, and provide a framework for considering future exciting, insightful and novel studies with direct clinical relevance.

3.2 Tubular Handing of Proteins

The main function of the kidney is to filter plasma, while at the same time, retain the majority of plasma proteins. The glomerular capillary wall has long been thought of as the major barrier to the passage of protein into the urine partly based on the fact that massive proteinuria results from genetic diseases of glomerular epithelial cells [1] and the very low amount of albumin measured in the filtered fluid by micropuncture studies [2]. However recent studies by many groups, including ours, have suggested that renal protein filtration under physiological condition is much greater than previously thought [3]. Of the many proteins present in the plasma albumin is the most abundant (~60 % of all plasma proteins) and the most studied. For this reason plasma protein and plasma albumin are quite often used interchangeably in the scientific literature.

Albumin is an anionic, 585 amino acid, single polypeptide chain with molecular weight ~67 kDa. Its physiological plasma concentration is 35–60 mg/mL. Though not essential to life, a number of important and diverse functions have been ascribed to albumin including—maintenance of oncotic pressure, regulation of fluid exchange across capillary walls, acid–base balance and transport of number of different substances including fatty acids, drugs, hormone, and vitamins. In a healthy person albumin is exclusively synthesized in the liver at a rate of 10–15 g/day. The half-life of albumin has been estimated to be 19 days which represents the balance between anabolism and catabolism, primarily within muscle, liver and kidney. The albumin in glomerular filtrate is largely taken up by the renal proximal tubular cells in an active process [4]. Preventing or reducing urinary albumin excretion thus makes the kidney a key player in "protecting" the organism from excessive loss of albumin and its ligands. Albumin loss in urine has long been used as a marker of kidney injury, whether it originates from glomerular dysfunction, defective PTC reabsorption, or a combination. Using various preclinical model systems, multiple investigative teams have shown that the PTCs, especially the S1 segment, have effective and efficient mechanisms of reabsorbing, transcytosing, and processing filtered albumin. Mechanisms for PTC uptake and metabolism of filtered albumin (Fig. 3.1) include receptor-mediated clathrin-dependent endocytosis and fluid-phase endocytosis. Two major cellular pathways appear to be involved in this process: the retrieval pathway and the degradation pathway. More than 95 % of the filtered albumin is taken up by the retrieval pathway and returned to the blood supply. A small amount, <5 % of filtered albumin, is targeted to lysosomes for degradation to smaller peptides which are exocytosed by PTC and ultimately excreted in urine.

3.3 PTC and Albumin Reabsorption

The initial report of glomerular filtered albumin returning to the renal vein was made through the introduction of small pulses of radioactive albumin into the artery or in the isolated perfused kidney, followed by examination of the radioactive profile from the renal vein effluent [5]. Intravital *in vivo* two-photon microscopy studies, which

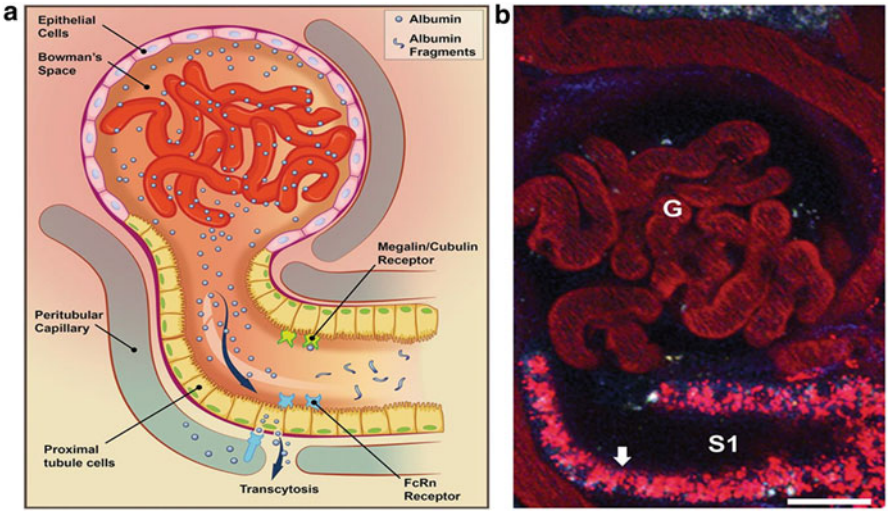

Fig. 3.1 Albumin filtration across the glomerulus is greater than previously thought and reclaimed by the PTC, especially S1 cells. (**a**) Albumin filtered at the level of the glomerular capillaries into the Bowman space is taken up after binding by the megalin-cubilin receptor complex or perhaps by the FcRn lining the brush border of proximal tubular cells. Albumin is internalized to PTCs by receptor-mediated endocytosis *via* clathrin-coated vesicles and fluid-phase endocytosis. From there it can be catabolized *via* lysosomal degradation or can be transcytosed. Albumin fragments in the urinary lumen result from lysosomal exocytosis or peptide hydrolysis by apical membrane proteases. (**b**) *In vivo* image of 25-micron three-dimensional volume showing amounts of Texas red–labeled albumin uptake into PTCs (*arrow*), especially the S-1 segment (S1). G, glomerular capillaries. Bar = 20 μm (Adopted from Landon E. Dickson et al. JASN 2014;25:443–53)

allow four-dimensional analysis (volume and time) of physiologic processes, permit direct visualization and quantification of glomerular filtration and quantitation of PTC uptake [6–8]. Using this method, evidence for the existence of the retrieval pathway *in vivo* has been recently provided in live Munich-Wistar Fromter (MWF) rats [3, 9]. MWF rats have many surface glomeruli, have been used in micropuncture studies, and spontaneously develop hypertension and progressive albuminuria [10, 11].

3.3.1 Endocytosis by Proximal Tubule

Apical bound membrane proteins megalin and cubilin, clustered into clathrin-coated pits, have been attributed to receptor-mediated endocytosis for uptake of cellular proteins and other molecules by endocytotic pathways. Depending on the cell type, coated pits make up between 0.4 % and 3.8 % of the cell's surface [12]. These pathways have been studied extensively, and numerous reviews exist [13, 14]. Two other mechanisms of protein internalization, caveolin-dependent internalization and fluid-phase endocytosis, have also been described and well studied. Using neutral fluorescent dextrans, markers of fluid-phase endocytosis, it has been shown that rapid cellular uptake of molecules,

which don't have receptor on the apical membrane, occurs in non-selective manner via fluid phase [15, 16]. The endocytic apparatus, clathrin-coated pits and vesicles, is found throughout the PT, although are notably fewer in the S3 segment [17]. As a result, protein reabsorption and degradation is greatest in the S1 segment of the PTCs and least in the S3 [18–20]. Kinetic studies of the rat PT have shown that internalization of cargo is highly active at the brush border. The membrane and trapped fluid (luminal fluid) contained in the apical membrane invaginations are internalized in very short time [21] thus large amount of luminal fluid is internalized via fluid phase endocytosis. This also correlates with the observation of regular cytoplasmic channels seen in the three-dimensional reconstruction of the two photon image containing fluorescent albumin [22]. Although the capacity of the retrieval pathway concurs with the high capacity/low affinity receptor as described by Maack [22], fluid phase endocytosis has not been yet quantified and its role in the albumin uptake by proximal tubular cells is a topic of intense debate and research interest. About 5% endocytosed albumin gets degraded within the lysosomes and regurgitated as albumin fragments [23]. Albumin degradation can occur in multiple sites. Degraded lysosomal albumin fragments were initially thought to be completely recycled back into circulation [22]. However most recent studies using isolated perfused rat kidneys [24], *in vitro* studies with HK-2 cells [25], and *in vivo* models with Sprague–Dawley rats, have shown that albumin can be rapidly degraded into small peptides and released back into the tubular fluid [20]. In the CD2AP knockout mouse high levels of intact albumin are found in the urine suggesting dysfunctional lysosomal PTC albumin degradation [26]. Using a microfluidic bioreactor it has been shown that fluid shear stress is an important factor mediating cellular protein handling by renal tubular epithelial cells in opossum kidney [27]. However, caution is required to extrapolate the *in-vitro* endocytosis data as *in vivo* PTCs have a rate of endocytosis that is far greater in magnitude than that of cultured PTCs [28]. In addition, the rate of apical endocytosis is many times that of basolateral endocytosis *in vivo,* but the two are equivalent in cell culture [29, 30].

3.3.2 The Megalin-Cubilin Complex

Originally identified as the antigen in Heymann nephritis (a model of membranous nephropathy) [31], megalin is an endocytic receptor belonging to low-density lipoprotein receptor family [32]. The extracellular domain contains four clusters of cysteine-rich, complement-type repeats, constituting the ligand binding regions. The ligand binding regions are separated by epidermal growth factor (EGF)-like repeats and cysteine-poor spacer regions containing YWTD motifs, so called propeller repeats, involved in pH-dependent dissociation of receptor and ligands in acidic endosomal compartments [33]. The cytoplasmic tail contains two NPXY motifs, which mediate the clustering in coated pits and thereby initiate the endocytic process. Cubilin is a 460-kDa peripheral membrane protein, previously referred to as gp280, and identical to the intrinsic factor-vitamin B_{12} receptor found in the small intestine [34]. Cubilin is composed of an initial 110-amino-acid region necessary for membrane anchoring of the receptor [35], followed by eight EGF-like repeats and 27 complement

subcomponents and bone morphogenic protein-1 (CUB) domains [36]. Many CUB domains of the cubilin receptor have ability to bind with variety of ligands.

Megalin and cubilin are highly expressed in the renal proximal tubule brush-border endocytic receptor complex as well as in the lysosomes [37, 38]. Apart from tubular cells, megalin is expressed in many extrarenal tissues like type II pneumocytes, thyroid and parathyroid cells, the choroid plexus, the endometrium, the oviduct, epididymis, ependymal cells, labyrinthic cells of the inner ear and the ciliary epithelium of the eye. The two receptors are co-localized in the proximal tubule, small intestine, visceral yolk sac and the cytotrophoblast of the placenta. The normal expression of megalin is dependent on receptor-associated protein (RAP) [39] serving as a chaperone to protect newly synthesized receptor from the early binding of ligands and possibly involved in folding of the receptor [40–42]. RAP binds megalin with high affinity within the endoplasmic reticulum and functions as an intracellular ligand inhibiting the binding of most other ligands to megalin. Decreased expression of either megalin or cubilin can result in number of diseases characterized by proteinuria [43–47].

Megalin and cubilin work in concert to reabsorb >40 filtered molecules [14, 48–50]. Although both are known to bind to albumin [51], cubilin is the major albumin binding protein and plays an important role in normal proximal tubule endocytic reabsorption of filtered albumin. Albumin binds to cubilin with a dissociation constant (K_d) of 0.63 μM at a pH of 7.0 [52] resulting in a high-affinity, low-capacity pathway of endocytosis that primarily targets product to the lysosome for degradation. Cubilin interacts with the transmembrane endocytic receptor megalin. Megalin's principal role seems to be in catalyzing the retrieval and internalization of apical cubilin-albumin complexes from glomerular filtrate. This multi receptor retrieval system is thought to have the capacity to process approximately 30–50 μg of albumin daily in mice [51]. Disruption of cubilin, megalin and/or cubilin-megalin complex results in proteinuria. In megalin knockout models, the internalization of endogenous ligands bound to apical cubilin, especially cubilin-albumin complexes, is markedly reduced. Albumin uptake in opossum kidney (OK) cells was inhibited by IF-B_{12}, and anti-cubilin antibodies [53]. Mice deficient in megalin in addition to cubilin did not exhibit any more albuminuria than mice with cubilin deficiency alone [54] suggesting that megalin's principal role is to facilitate cubilin-albumin internalization. In dab2 (the protein involved in coated pit formation) knockout mice coated pits were not formed resulting in dysfunctional endocytosis and proteinuria [55]. Recently it has been shown that proximal tubules have the capacity to regulate the uptake of albumin [56].

3.3.3 FcRn Receptor

The neonatal Fc receptor (FcRn) was discovered by Jones and Waldman as a heterodimeric membrane bound protein with class I MHC-like properties containing a heavy chain and β_2-microglobulin like light chain [57]. They found that this receptor could bind to specific IgG molecules and aid in their intestinal uptake and transport. Wild-type FcRn has two distinct and separate binding sites for albumin and IgG [58]. Binding of

albumin and IgG is low affinity and high capacity at a physiologic pH, with increasing affinity occurring at a lower pH. In humans, FcRn is derived from the *FCGRT* gene encoded on chromosome 19 located outside of the MHC class I locus on chromosome 6. Rat and mice FcRns are 91% identical, and both are encoded on chromosome 7. Human FcRn has one *N*-glycan moiety and its molecular mass is approximately 42–44 kD, while the molecular mass of rat FcRn is 48–52 kD which is attributed to three additional *N*-glycan moieties [59]. Within the kidney, FcRn is found in the vascular endothelium, podocytes, cortical collecting duct and proximal tubular epithelial cells [60]. Apart from kidney it is also found in epithelial cells of small intestine, liver, spleen, lung, placental syncytiotrophoblasts, polymorphonuclear neutrophils, monocytes, phagocytes and dendritic cells [61–65]. FcRn mediates transcellular IgG transport in maternal milk during lactation to the newborn [66, 67]. In the small intestine FcRn plays important role in IgG endocytosis *via* clathrin-coated pits at low luminal pH [68].

In the kidney FcRn role appears to be that of intracellular selection, sorting, and preservation of reabsorbed albumin and IgG. It remains to be determined whether FcRn participates in luminal albumin binding, although this is not favored by the luminal pH. However, the megalin-cubilin bound albumin within the clathrin-coated pits, and fluid-phase endocytosis vesicles undergo pH reduction to approximately 5.0. At the low pH found in endosomes, albumin dissociates from megalin-cubilin, while FcRn's affinity to bind both IgG and albumin increases dramatically [58, 64, 69, 70]. Thus, albumin is capable of moving from a low-capacity lysosomal degradation pathway [71, 72] to a high-capacity pathway of FcRn-mediated transcytosis and recycling based on inherent binding properties of the receptors [5, 73, 74]. Binding studies have shown that FcRn has a single binding site for albumin that is distinct from the IgG site and that both these interactions are pH dependent. The equilibrium dissociation constant, K_d, is much weaker at a pH of 7.0 (34–408 μM) versus a pH of 5.0 (0.2–0.7 μM) [58]. Consequently, if albumin is internalized while bound to the megalin-cubulin complex and is trafficked to the late endosomes, it encounters acidic pH and a "handoff" of albumin to the FcRn receptor can occur, thus directing it down the transcytotic pathway. When the transcytotic vesicle fuses with the plasma membrane and encounters neutral physiologic pH, a rapid dissociation of albumin from FcRn will occur, thereby releasing it to the interstitium and ultimately back into the circulation *via* the FcRn-mediated pathway in the endothelium [70, 74–76]. The FcRn receptor is recycled back to the apical membrane or apical compartment, ready for another cycle of albumin transcytosis. Of critical importance for albumin dynamics may be how modified albumins (*i.e.*, glycated, carbamylated, and various drugs bound to albumin) affect the albumin-FcRn pH-dependent binding interaction. For instance, increased binding at a neutral pH or decreased binding at an acidic pH may both result in more targeting to lysosomes (Fig. 3.2). The first direct evidence for transcytosis of albumin came from PT microperfusion studies [22]. Subsequent studies, using transmission electron microscopy immunogold technique, revealed albumin uptake across the apical membrane and release across the basolateral membrane of PTCs [3]. Subsequent two-photon studies showed actual intracellular vesicles and tubules uniting with the basolateral membrane and releasing fluorescently labeled albumin into the interstitium [77]. Tenten et al. [9] showed that both negatively charged and neutral albumin released from transgenic podocytes was transcytosed from the filtrate into the blood. Furthermore, genetic deletion of the FcRn receptor in these

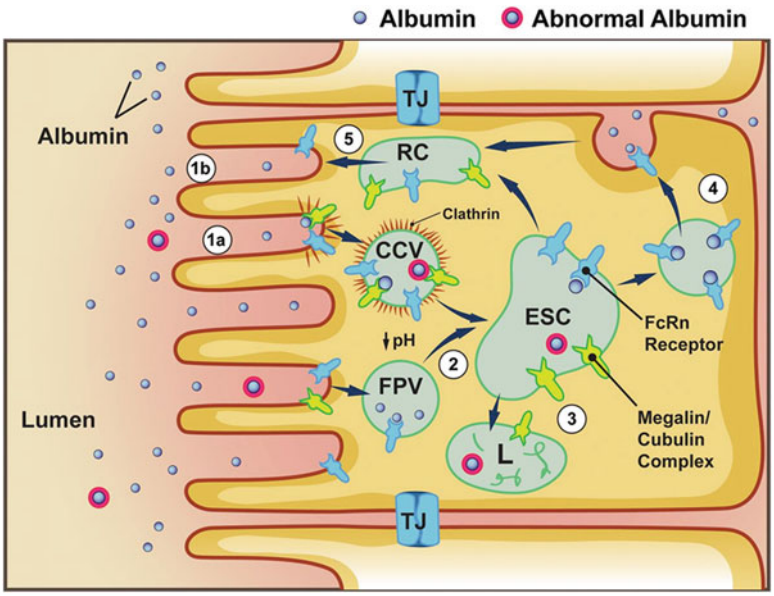

Fig. 3.2 FcRn mediates pH-dependent transcytosis and intracellular sorting of reabsorbed albumin. Albumin is reabsorbed *via* both receptor-mediated clathrin-coated pits into vesicles (CCV) (*1a*) and by fluid-phase (clathrin-negative) endocytosis (*1b*). Following endocytosis, endosomal acidification occurs (*2*), causing dissociation of albumin from receptors, such as megalin-cubilin complexes. However, acidification enhances albumin binding to FcRn throughout endocytic compartments; thus, there is exchange of albumin from the megalin-cubulin complex to FcRn. Within the endosomal-sorting compartment (ESC), albumin is directed toward lysosomal degradation or the transcytotic pathway (*3*). Transcytosis occurs by both vascular and tubular structures mediating albumin delivery to the basolateral membrane (*4*). Upon fusion with the basolateral membrane, the increase in pH of the extracellular environment causes dissociation of albumin from FcRn; FcRn is then recycled back to the apical membrane *via* the recycling compartment. It is possible that albumin's binding to FcRn is reduced by alterations, such as glycosylation and carbamylation; thus, transcytosis of albumin would not occur and albumin would enter the lysosomal pathway. This would provide an intracellular molecular sorting mechanism to preserve physiologic albumin and facilitate catabolism of chemically altered albumin. FPV, fluid-phase vesicle; L, lysosome; RC, recycling compartment; TJ, tight junction (Adopted from Landon E. Dickson et al. JASN 2014;25:443–53)

mice abolished transcytosis of both types of albumin. These data prove that FcRn is responsible for mediating albumin transcytosis in the PTC. However, the magnitude of this process remains to be determined.

3.4 Dysfunctional PTC and Proteinuria

Proteins are well known to be reabsorbed by proximal tubular cells [22]. To increase the plasma half life of the most abundant protein, albumin, mammals have developed multiple cellular mechanisms for minimizing albumin turnover [78]. Individual disruption of numerous specific PTC processes have been documented to cause

Table 3.1 Data implicating role for the PT in albumin processing and/or albuminaria

Process implicated or defective	Reference
D-serine–induced PTC injury	Carone and Ganote [79]
Megalin-cubilin complex	Birn et al. [39, 52]; Christensen and Birn [48]
ClC-5 knockout	Piwon et al. [45]; Christensen et al. [80]
Total-body irradiation	Yammani et al. [81]
NHE-3 knockout	Gekle et al. [82]
Statins	Sidaway et al. [83]; Verhulst et al. [84]; Atthobari et al. [85]
Rab 38	Rangel-Filho et al. [86, 87]
Increased GSCs	Russo et al. [3]
Transcytosis	Russo et al. [3]; Sandoval et al. [77]
FcRn	Sarav et al. [69]
Carbon nanotubes	Ruggiero et al. [88]
Bardoxolone	Reisman et al. [89]
Diphtheria toxin–induced PTC injury	Grgic et al. [90]; Sekine et al. [91]; Zhang et al. [92]

proteinuria. Selective defects occurring in the involved tubular transport processes and their quantitative importance are tabulated in Table 3.1. Cubilin, a 460 kDa receptor, heavily expressed in the kidney proximal tubule is known to bind with albumin [52]. Another protein, megalin, plays a crucial role in protein reabsorption by catalyzing the retrieval and internalization of apical cubilin-albumin complexes from glomerular filtrate [48, 93, 94]. Defects in either of these two endocytic receptor complex proteins, megalin and cubilin, yield increased levels of albuminuria, suggesting a role in albumin reabsorption and metabolism. Bardoxolone methyl, a potent activator of the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant and anti-inflammatory response, is known to cause significant albuminuria by decreasing renal expression of megalin but not cubilin [89]. Single dose of total-body irradiation in rats resulted in total loss of the ability of albumin and megalin to bind to cubilin, resulting in albuminuria [81]. Pharmacological and genetic studies in cultured opossum kidney cells (OK cells) have shown that the apical Na(+)/H(+) exchanger isoform 3 (NHE3) supports receptor mediated endocytosis by interference with endosomal pH homeostasis and endocytic fusion events. NHE3 exchanger also supports proximal tubular protein reabsorption in vivo [82]. Proteinuria was observed in mice lacking renal chloride channel, CLC-5, required for endosomal acidification and trafficking, is associated with defective receptor-mediated endocytosis and fluid-phase endocytosis [95, 96]. Defective endocytosis in *ClC-5* knockout mice is now known to be due to trafficking defects related to selective loss of brush-border cubilin and megalin, causing albuminuria [80]. Rab38, a gene having a causal role in determining the phenotype of the fawn-hooded hypertensive rat, modulates proteinuria through effects on tubular re uptake and not by altering glomerular permeability [86]. In opossum kidney cells, receptor-mediated protein endocytosis is reduced by statins, inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which are widely used for therapeutic

reduction of plasma cholesterol levels thus giving rise to proteinuria in some patients [84]. Another statin, Simvastatin, inhibited receptor-mediated endocytosis of both FITC-albumin and FITC-β2-microglobulin to a similar extent but without altering the binding of albumin to the cell surface [83]. The reduction in albumin uptake was also related to the degree of inhibition of HMG-CoA reductase. A random control trial (RCT) showed that statins may inhibit guanosine triphosphatase prenylation, which reduces proximal tubular endocytosis thus enhancing proteinuria [85]. Administration of D-serine to rats induced acute necrosis of the proximal straight tubules, proteinuria, glucosuria, and aminoaciduria. Proteinuria and glucosuria developed at the onset of tubular necrosis and disappeared when the tubules were completely relined by new epithelium [79]. Three independent studies have shown that diphtheria toxin (DT) selectively depletes mouse kidney proximal straight tubule [91] causing acute kidney injury (AKI) leading to nephrotic range albuminuria without associated glomerular morphologic injury, [90, 92]. This extensive proteinuria resolved with regeneration of intact and functional PT cells.

3.5 Proteinuria Induced Tubulo-Fibrogenesis

Proteinuria has been show to induce tubulo-fibrogenesis of albumin induced in cultured proximal tubular cell. TGFβ is a profibrogenic cytokine capable of directly stimulating the proliferation of fibroblasts and the synthesis of matrix proteins, in addition to exerting indirect stimulatory effects via inflammatory infiltrating cells. TGFβ acts as a key stimulus for epithelial- to-mesenchymal transition (EMT), by which tubular cells acquire features of the fibroblast [97]. Stahl's group have shown albumin upregulated ligand-binding TGFβ receptors on cultured proximal tubular cells which became more susceptible to the matrix-stimulatory actions of TGFβ [98]. Albumin stimulated the accumulation of extracellular collagen type IV, laminin, and fibronectin by proximal tubular cells through a post- transcriptional mechanism [99]. Recent studies have shown that up-regulation of the kinin B2 receptor pathway modulates the TGF-β/Smad signaling cascade to reduce renal fibrosis induced by albumin [100]. Reduced degradation could be responsible for the increased accumulation of extracellular matrix protein components, as indicated by induction of tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2, in response to albumin [99]. Interstitial fibrosis represents the final common pathway of any form of progressive renal disease. It is a well established fact that fibrosis generating myofibroblasts and activated matrix secreting cells are the hallmark of the process [101–104]. In proteinuric settings, protein overload and reabsorption by proximal tubular cells initiate or enhance fibrogenesis by at least two mechanisms. First, proximal tubular epithelial cells have the potential to interact directly with the adjacent interstitial fibroblasts via paracrine mechanisms. Proximal tubular cells can synthesize platelet derived growth factor (PDGF) and TGFβ1 and stimulate renal cortical fibroblasts in co-culture to synthesize collagen [104]. Second, the proinflammatory activation of tubular cells fosters local recruitment of macrophages and lymphocytes that by releasing TGFβ, PDGF and other cytokines to stimulate interstitial cells to produce excess matrix [105]. The tubular paracrine pathway and the inflammatory cell-mediated

pathway are activated after the onset of proteinuria. Cells expressing the myofibroblast associated marker α-smooth muscle actin (α-SMA) were detected in interstitial areas and colocalized with macrophages surrounding proximal tubular cells that were engaged in excess protein reabsorption [106]. In addition to the activation of interstitial cells, the fibrogenic reaction involves a phenotypic reversal of tubular epithelial cells [102]. A number of studies have documented the abnormal expression of α-SMA and other myofibroblast markers in renal tubule epithelial cells both in human and experimental nephropathies [107]. Urinary proteins from nephrotic patients with focal segmental sclerosis, or to a lesser extent in patients with minimal change disease, induced cultured proximal tubular cells to express EMT-related patterns including α-SMA and vimentin via ERK1/2 and p38 pathway [108]. TGFβ remains the most important cytokine for renal fibrogenesis. It has also been identified as the best characterized stimulus for EMT in renal tubular cells. Studies have focused on the signaling pathways which are activated during TGFβ-induced EMT. TGFβ caused Smad2 phosphorylation in a tubular epithelial cell line, and overexpression of the inhibitory Smad protein, Smad7, inhibited TGFβ 2 induced Smad2 activation, thereby preventing EMT and collagen synthesis [109, 110]. An endogenous antagonist of TGFβ1-induced EMT has been identified as bone morphogenic protein-7 (BMP-7), a member of TGFβ superfamily whose genetic deletion in mice leads to severe impairment of kidney development [111]. Systemic administration of recombinant BMP-7 repaired severely damaged renal tubular epithelial cells and reversed renal injury in mice with nephrotoxic serum nephritis [112]. Other mediators that may critically contribute to fibrogenesis include PDGF [113] and endothelin-1 [114] which are able to activate α-SMA gene expression in renal fibroblasts and vascular smooth muscle cells, respectively. Thus, mounting evidence indicates that glomerular proinflammatory cytokines combined with massive proteinuria are major determinants of subsequent tubulo-interstitial injury and progressive kidney failure in experimental and human nephropathies.

3.6 Proteinuria and Tubular Apoptosis

Protein overload is a stimulus for apoptosis. A dose- and time-dependent induction of apoptosis by albumin was demonstrated in cultured proximal tubular cells as revealed by internucleosomal DNA fragmentation, morphological changes including cell shrinkage and nuclear condensation, and plasma membrane alterations [115]. Apoptosis in this case was associated with activation of Fas-FADD-caspase 8 pathway, suggesting activation via the extrinsic pathway of apoptosis. Peroxisome proliferator activated receptor (PPAR)-γ is also implicated in molecular mechanisms underlying albumin-induced apoptosis [116]. In HKC-8 human proximal tubular cells, albumin-induced apoptosis was mainly mediated by the intrinsic pathway of apoptosis, characterized by Bax translocation to mitochondria and cytochrome c release from the organelles [117]. Recently brain abundant signal protein 1 (BASP1) was shown to modulate albumin induced apoptosis in tubular cells [118]. Albumin-dependent signaling and albumin endocytosis appear to act as interrelated processes regulating the fate of proximal tubular cells

in vitro. It has been suggested that megalin may behave as a sensor molecule that determines whether the cells will be protected from or injured by albumin, depending on the protein concentration. On one hand, low concentrations of albumin lead to activation of the serine/threonine kinase PKB and phosphorylation of BAD protein, which inhibits apoptosis [119]. On the other hand, albumin overload decreased the expression of megalin on the plasma membrane that was associated with a reduction of PKB activity and BAD phosphorylation, favoring apoptosis [120]. In albumin overload models of tubular cells, a balance has been suggested between the induction of a pro-inflammatory NF-kB dependent, Bcl-xL mediated anti- apoptotic pathway and the induction of AP-1 mediated clusterin overexpression that, by inhibiting the inflammatory pathway, would favor a switch from an inflammatory phenotype to apoptotic injury [121]. It has been suggested that persistent proteinuria causes apoptosis in tubular cells through the activation of AT2 receptor, which can, in turn, inhibit MAP kinase (ERK1/2) activation and Bcl-2 phosphorylation [122]. Multiple pathways of apoptosis can be activated in renal tubular cells during proteinuric kidney diseases. Apoptotic responses to protein load were documented in the rat model of albumin overload proteinuria, showing increased numbers of terminal dUTP nick- end labeling positive apoptotic cells both in the tubulointerstitial compartment and in glomeruli [122]. Proximal tubular cell apoptosis may contribute to glomerular-tubule disconnection and atrophy in response to proteinuria in rats with accelerated passive Heymann nephritis [123]. Renal tubular cells exposed to a high protein load suffer from endoplasmic reticulum (ER) stress which may subsequently lead to tubular damage by activation of caspase-12 [124]. Apoptotic cells were also detected both in proximal and distal tubular profiles in biopsy specimens of patients with primary focal segmental glomerulosclerosis. A positive correlation was found between proteinuria and incidence of tubular cell apoptosis, which was identified as a strong predictor of outcome in these patients [125]. Besides promoting tubulo-glomerular disconnection at the proximal level, tubular apoptosis could create and sustain a local proinflammatory microenvironment via release of molecules that serve as danger signals by dying cells. Danger-associated molecular patterns (DAMPs) trigger inflammation by engaging Toll-like receptors (TLR) and nucleotides-binding domains, leucin-rich, repeat-containing proteins (NLRs). Engaged NLR form complexes with apoptosis- associated proteins to produce macromolecular complexes termed inflammasomes that cleave proinflammatory cytokines to their mature forms [126]. Albumin has been show to activate NLRP3 inflammasome in both *in vitro* renal tubular cells and *in vivo* kidneys in parallel with significant epithelial cell phenotypic alteration and cell apoptosis. Genetic disruption of NLRP3 inflammasome attenuates albumin-induced cell apoptosis and phenotypic changes under both *in vitro* and *in vivo* conditions. Also, albuminuria results in a significant mitochondrial abnormality as evidenced by the impaired function and morphology, which was markedly reversed by inhibition of theof NLRP3/caspase-1 signaling pathway [127, 128]. In cultured proximal tubular cells albumin dose-dependently enhanced NF-kB activity resulting in upregulation of RANTES, MCP-1 and IL-8 [129–131].

3.7 Summary

Although the contribution of proximal tubule dysfunction to proteinuria is a topic of intense debate and research, mounting evidence points toward the undeniable role of the proximal tubule in albumin reabsorption and reclamation. Many single-site mutations and complete PTC dysfunction result in a high level of albuminuria, without any histologic or electron microscopy structural alterations in the glomerular filtration barrier. Reabsorption of filtered albumin involves a low-capacity/high-affinity megalin-cubulin receptor-mediated process and a high-capacity/low-affinity, process that could be fluid-phase endocytosis. Recent papers strongly suggest a role forthe FcRn receptor in albumin binding, sorting and intracellular trafficking between transcytosis and degradation pathways in a pH dependent manner. Future studies are warranted examining proteinuria not only as a glomerular impairment but also as proximal tubule dysfunction and may lead to many new advances in the diagnosis and treatment of proteinuric states.

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Chapter 4
Pathophysiology of Diabetic Nephropathy

Michal Herman-Edelstein and Sonia Q. Doi

Abbreviations

ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic hormone
AGEs	Advanced glycation end products
AKI	Acute kidney injury
ARBs	Angiotensin II receptor blockers
ATF6	Activating transcription factor 6
CKD	Chronic kidney diseases
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
DKD	Diabetic kidney disease
DN	Diabetic nephropathy
eGFR	Estimated glomerular filtration rate
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ESRD	End-stage renal disease
ET-1	Endothelin Receptor Antagonists
GBM	Glomerular basement membrane
IRE1	Inositol requiring enzyme 1
MCR	Melanocortin receptors

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MMPs	Matrix metalloproteinases
NF-kB	Nuclear factor Kappa B
PERK	Protein-kinase-RNA-like ER kinase
RAGE	Receptors for AGE
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
T2DM	Type 2 diabetes
UAE	Urinary albumin excretion
α-SMA	α-Smooth muscle actin

4.1 Diabetic Nephropathy: Clinical Manifestations

The increasing prevalence of diabetic kidney disease (DKD) is primarily due to the increased prevalence of obesity and type 2 diabetes (T2DM) worldwide. DKD is the leading cause of end-stage renal disease (ESRD), accounting for approximately 50 % of ESRD cases, thus representing a major health concern worldwide [1–5].

In spite of all the beneficial secondary prevention interventions for patients with DKD, including tight glucose control and tight blood pressure control with regimens that inhibit the renin angiotensin aldosterone system (RAAS), renal disease still progresses in most of these patients.

Currently, microalbuminuria is considered to be the first clinical evidence for new diabetic nephropathy (DN). Typically, onset of diabetic nephropathy (DN) is considered when microalbuminuria appears, and an increase in albuminuria can predict risk for disease progression, as well as be a marker of increased risk for cardiovascular morbidity and mortality [6–8].

Albuminuria is a marker for all renal and mainly glomerular diseases, including diabetic nephropathy. Albuminuria plays an important role in the pathogenesis of diabetic nephropathy. It is commonly used in the clinic as a tool for predicting prognosis and monitoring response to therapy, but its strength as a marker is limited [9].

Diabetic nephropathy has been didactically divided into stages based on the values of urinary albumin excretion (UAE): a normoalbuminuria group (UAE < 30 mg/g creatinine on a random urine sample), a microalbuminuria group (UAE 30 ~ 300 mg/g), and a macroalbuminuria group (UAE > 300 mg/g). Microalbuminuria is an early sign of renal microvascular disease in diabetes, and is a predictor of cardiovascular disease, development of DN and early mortality [8, 10, 11].

Persistent albuminuria (>300 mg/24 h or 200 μg/min) is the hallmark of irreversible nephropathy, a key sign in the clinical criteria for diagnosis of DKD [12].

Overt proteinuria or macroalbuminuria predict progression of kidney disease in both type 2 diabetes mellitus in PIMA Indians [13] and in T1DM [14]. Once macroalbuminuria occurs, glomerular filtration falls rapidly at a rate of 2–20 mL/min per year [15].

The urine sediment in diabetic nephropathy is traditionally described as bland proteinuria, but microscopic hematuria commonly occurs in 48 % of biopsy proven diabetic nephropathy (DN) [5, 6], with a possible association between the disease severity and hematuria [7].

Most of the classical data on the natural clinical course of DN were obtained before the era of tight glycemic control, aggressive blood pressure control, lipid control and the benefit of angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs).

This historical data does not necessarily represent the natural history of today's DKD. Over recent years there has been encouraging improvement in the course and outcome of DN with decreased incidence of new ESRD and cardiovascular mortality. Patients who are well treated may have relatively stable renal function or a very slow rate of progression [16, 17].

The progressive natural history of diabetic kidney disease with type I diabetes was primarily described in the 1993 Captopril Collaborative Study Group, showing a 17 % rate of decline in creatinine clearance per year without ACE inhibitors in patients with type I diabetes [18]. Recently, with improved standard glycemic control in T1DM, a study from Sweden has noted a dramatic reduction in clinically evident diabetic nephropathy to only 8.9 % in 25 years [19].

The same improvement was also seen in T2DN in the 1990s' first NIH study, where nephropathy developed in up to 50 % of young diabetic Pima Indians, with 15 % progression to ESRD. However, with the use of modern therapies the incidence of ESRD, even in this group of extremely high risk for diabetes complications, declined significantly from the period 1991–1994 to the period 1999–2002 (32 to 15 cases per 1000 patient-years, respectively) with a rate of fall in GFR in the last group of 0.93 mL/min per month [20, 21].

The classical course of the disease and its progression was described as following from microalbuminuria to macroalbuminuria and progressive decline in glomerular filtration rate (GFR) [10]. This clinical paradigm has recently been questioned as in large epidemiologic studies, renal insufficiency has developed before the onset of overt proteinuria or without a microalbuminuric stage [22–26].

Important data relating the rate and clinical history of development of diabetic nephropathy in a population of predominantly white patients with newly diagnosed T2DM is reported in the United Kingdom Prospective Diabetes Study (UKPDS). Fifteen years after diagnosis, 38 % of 4,031 UKPDS participants developed albuminuria and 29 % developed renal impairment, 575 (51 %) of the latter did not have preceding microalbuminuria [27].

In our study on patients with biopsy-proven diabetic nephropathy, eGFR deteriorated at a rate of 1.0 ± 0.9 mL/min/1.73 m^2/month (excluding patients who started dialysis in the 2 months after biopsy). Patients reached dialysis within 29.7 ± 23.6 months after kidney biopsy [28–30].

The level of proteinuria in DN is not a reliable surrogate marker for declining kidney function [4, 9]. The limitations of using albuminuria or GFR alone are apparent, with their use as trial endpoints requiring further thought. Impaired kidney function was found frequently in asymptomatic, normotensive, non-albuminuric patients with T2DM and there is no direct correlation between the level of proteinuria and the level of eGFR. In the UK Prospective Diabetes Study (UKPDS), 51 % of those who developed an estimated creatinine clearance of less than 60 mL/min/1.73 m^2 never tested positive for albuminuria [31].

The relationship between renal pathological structural changes and the level of proteinuria is very complex, and incompletely understood [32]. Widening of the

glomerular basement membrane (GBM), a hallmark feature of diabetic pathology, can also be seen in non albuminuric T1DM patients [33]. Furthermore, baseline albuminuria during study enrollment does not always correlate with the course and progression of eGFR decline in T2DN [25, 26, 33, 34].

Type 2 diabetic patients are diagnosed at varying time points in their disease including late in the course of diabetes. Recently, Perkins *et al.* reported that in patients with T1DN, who are always diagnosed early and are in regular follow-up for albuminuria, GFR loss develops before proteinuria [32, 35].

Longitudinal follow-up studies show that persistent microalbuminuria in the DCCT/EDIC cohort was not a good predictor for progression; 40% of T1DM patients who developed persistent microalbuminuria "regressed" to normoalbuminuria and did not progress to macroalbuminuria or ESRD within 10 years [16].

Discordance appears between progressive changes in albuminuria and creatinine doubling time in a series of clinical trials in T2DN [27]. For example, in the Action to Control Cardiovascular Risk in Diabetes (ACCORD), reduction of albuminuria did not correlate with the risk of doubling of serum creatinine [36].

Hyperfiltration is widely regarded as a contributing factor in the development of microalbuminuria and progressive nephropathy in T1DM, since it elevates intraglomerular pressure [37]. However, in a larger FinnDiane cohort (n = 2168 patients) [19], a long-term follow-up of patients with hyperfiltration, eGFR levels in 90th percentile were not associated with microalbuminuria [19, 38, 39]. The hyperfiltration is partially related to poor metabolic control, and intensification of glycemic control can reduce GFR towards normal.

Methods used for albuminuria measurement are not fully standardized and that can also explain in part the low predictive value of albuminuria as a biomarker. Furthermore, there are daily variations in albuminuria of 40% for those with T1DM and an ACR of 30–300 mg/g creatinine. Albuminuria may also be increased by episodic hyperglycemia, high blood pressure (BP), high-protein diet, exercise, fever, urinary tract infection and congestive heart failure. Recommendations from the NKF and National Kidney Disease Education Program (NKDEP) support measuring albuminuria more than once and state that two of three samples should be elevated over a 3–6-month period for confirmation of a diagnosis of increased albuminuria.

Although kidney disease attributable to DN is very common in T2DM, patients with diabetes may have other etiologies of chronic kidney disease in addition to diabetes and they should have full clinical and laboratory assessment. Although the gold standard for the diagnosis of DKD is renal biopsy, diagnosis of DKD is made based on clinical grounds. Biopsy should be considered when diagnosis is unclear. Clinical diagnostic criteria for DN include: (1) long history of diabetes; (2) presence of diabetic retinopathy and/or neuropathy; (3) persistent proteinuria or albuminuria; (4) no severe hematuria and/or cellular casts; (5) increase of GFR with enlargement of kidneys; (6) all patients should undergo complete evaluation for other kidney diseases [9, 12, 40].

Renal biopsy is indicated in diabetic patients with an atypical presentation of renal disease that could be attributed to other renal disease [41, 42], including microalbuminuria without diabetic retinopathy, rapid decline of glomerular filtration rate, rapid increase of proteinuria, sudden appearance of the nephrotic syndrome, active sediment or the appearance of signs and symptoms of other systemic diseases.

The challenge of diagnosis and management of diabetic kidney disease and the absence of good biomarkers should lead us to a more frequent use of renal pathology in the management of the young patient [15]. The presence of diabetic retinopathy in patients with proteinuria strongly predicts the presence of DKD [43]; however, the association is not strong in early diabetes.

A major factor that may contribute to the development of ESRD in patients with diabetes is acute kidney injury (AKI). AKI episodes cause faster progression of renal failure [44]. These episodes are usually irreversible in the diabetic kidney and should be prevented.

Early identification of patients at risk for incidence or progression to end-stage renal failure by the use biomarkers is highly desirable. Along with albuminuria and GFR, new sensitive biomarkers in the serum or urine should be studied and validated [45, 46]. These diagnostic and prognostic biomarkers of renal disease will potentially be used to study the effect of different therapy interventions [47, 48].

4.2 Pathophysiology of Diabetic Nephropathy

A number of hemodynamic, metabolic, cellular and molecular changes contribute to the manifestations of chronic kidney diseases (CKD). In diabetic nephropathy (DN) particularly, most of these alterations primarily originate in response to prolonged hyperglycemia, and may in turn induce secondary pathological changes [49]. With the advance of scientific methodologies, the intricate network of pathophysiological processes underlying the development of DN has been expanding in molecular details. Although some factors contributing to the development of DN may occur simultaneously and are interdependent, for didactic purposes they will be discussed separately.

4.2.1 Hemodynamic Changes

Histologically, DN is characterized by an increased content of extracellular matrix (ECM) in the glomerulus and interstitium. The progressive accumulation of scar tissue in the glomerulus (glomerulosclerosis) and arterioles (arteriolosclerosis) results in loss of blood flow autoregulation and in reduced glomerular perfusion. Conversely, there is evidence that early hemodynamic changes precede glomerular fibrosis and play a role in initiating the development of DN [50–52].

Glomerular hyperfiltration has been documented since the early 50's in individuals at the onset of diabetes with poor metabolic control [53–55]. The increased glomerular filtration rate has been associated with changes in efferent and particularly afferent arteriolar resistance leading to an increase in the glomerular capillary plasma flow and in mean glomerular capillary pressure [51, 56]. Several additional factors may contribute to hyperfiltration, including vasoactive agents (renin-angiotensin system, nitric oxide, cyclo-oxygenase-2 derivatives), increased sodium reabsorption in proximal tubules, hormones (atrial natriuretic peptide, glucagon, insulin-like factor-1) and systemic hypertension [57, 58].

The hypothesis that implicates hyperfiltration as the initial factor in the development of DN is based on the argument that an increase in glomerular pressure causes an increase in single-nephron filtration rate, with irreversible damage to some glomeruli, and consequent redistribution of blood resulting in overburden of the remaining functional glomeruli. It has been demonstrated that a significant increase in glomerular pressure causes mechanical stress that contributes to mesangial cell damage and podocyte effacement resulting in ECM accumulation and albuminuria [59, 60]. However, the causal role of hyperfiltration in chronic kidney disease is still unclear [57, 58].

4.2.2 Kidney Fibrosis

Fibrosis results from a wound-healing mechanism that persists in response to continuous injury. In DN, both glomerular and tubulointerstitial fibrosis occur with accumulation of ECM that impairs the glomerular filtration and may progress to complete loss of kidney function.

In nodular glomerulosclerosis, a hallmark of DN, the accumulated ECM is rich in collagen types I and III, which are present in low abundance in normal kidney, and are therefore considered pathological. In addition, the ECM contains an increased amount of normal capillary basement membrane components, including collagen IV and V, fibronectin, laminin, perlecan and heparin [61].

Myofibroblasts, spindle-shaped cells expressing α-smooth muscle actin (α-SMA) have been implicated as the major source of ECM in the diabetic glomerular and tubulointerstitial fibrosis [61–63]. In comparison to fibroblasts, the myofibroblasts exert a higher contractile force, produce extracellular matrix proteins more abundantly, and can distort the renal architecture [61].

Following an initial insult, leukocytes and macrophages infiltrate the glomerular area releasing pro-inflammatory cytokines and growth factors that activate resident cells. The first cells recognized to undergo activation and phenotypic transformation to myofibroblasts were fibroblasts [64] and mesangial cells [65]. Later, podocytes [66] and parietal cells [67] were also documented to be activated and increase production of ECM proteins, contributing to the expansion of mesangium, thickening of the glomerular basement membrane and of the Bowman's capsule. Increasing evidence suggests that podocytes and PECs are transcriptionally reprogrammed late in development to acquire more mesenchymal or pericyte-like characteristics [61].

The source of the interstitial myofibroblast in CKD is still controversial. Some advocate that the vast majority of myofibroblasts are derived from nephrogenic progenitors, while others suggest that myofibroblasts derive from multiple cellular sources, including stroma, endothelium, leukocytes and epithelium [61, 68]. According to a review of the literature, in addition to originating from resident and bone-marrow derived fibroblasts, myofibroblasts may arise from epithelial-to-mesenchymal transition (EMT) of podocytes and tubular epithelial cells, and also from endothelial cells (EndoMT) and mesangial transdifferentiation [69]. In CKD progression, myofibroblasts synthesize matrix proteins, release reactive oxygen species and inflammatory cytokines [16, 19, 67].

4.2.3 Metalloproteinases and ECM

As discussed above, activated glomerular and interstitial cells contribute to the increased accumulation of ECM proteins. Abnormal accumulation of ECM in glomerulosclerosis results from an imbalance between synthesis and degradation of matrix glycoproteins by matrix metalloproteinases (MMPs). Activity of MMPs may in turn, be regulated by its transcription, translation and post-translational processes, in addition to tissue metalloproteinase inhibitors (TIMPs).

A number of studies reported altered expression of MMPs and TIMPs in DN. Data from clinical studies were somewhat conflicting [70]. However, some of the disparities may be related to the methodology employed, difference in samples, and individual variations. For example, in kidney tissue of diabetic subjects, one study reported decreased mRNA expression of MMP2 [71], while another reported an increase in protein expression and activity of this metalloproteinase [72]. In animal models of DN, there were findings of decreased expression and activity of MMP2 and MMP9, as well as increased expression and activity of TIMP2. However, depending of the animal model results may also vary [73]. Despite these variations, it is widely accepted that MMPs and TIMPs play a critical role in renal fibrosis. MMP2 concentration measured in the urine of diabetic individuals correlated with several clinical parameters including renal hyperfiltration and albuminuria, suggesting that MMPs and TIMPs may be clinically relevant as biomarkers of DN [70].

4.2.4 Advanced Glycation End-Products

Hyperglycemia induces nonenzymatic glycation of proteins and lipids, with the irreversible formation of reactive advanced glycation end products (AGEs). Accumulation of AGEs in kidney tissue has been associated with various deleterious effects observed in glomeruli, including increased vascular permeability, elevated synthesis and release of cytokines and growth factors, and up-regulation of matrix proteins [74–77].

AGE-induced damage in diabetic nephropathy may result from structural and functional alteration of extracellular proteins and from cellular responses mediated through receptors [78, 79]. The interaction of AGEs with the receptors for AGE (RAGE) induces a number of damaging effects that may be differentially regulated in diabetic nephropathy compared to non-diabetic renal diseases [79–81]. In diabetic nephropathy, an increased RAGE expression has been found in glomerular endothelial and mesangial cells and in podocytes [80, 81]. It has been suggested that the AGE-RAGE interaction in podocytes plays a key role in the induction of oxidative stress and inflammatory responses in the pathogenesis of DN [82, 83].

RAGE belong to the pattern recognition receptor family and therefore binds numerous damage-associated molecular patterns (DAMPs) molecules and a chronic RAGE activation may perpetuate the inflammatory response inducing fibrosis [84]. In addition, AGE-RAGE interaction increases ROS generation and subsequent activation of MAPK/p42/44 and NF-kB, resulting in an enhanced expression of RAGE in a positive autoregulatory loop [83].

In vitro and in vivo studies demonstrate clearly that activation of RAGE plays a major role in the onset and progression DN. Triggering of inflammatory effector mechanisms (generation of cytokines and chemokines, and expression of cell adhesion molecules) mediated by the AGE–RAGE interaction involves multiple intracellular signal transduction pathways, including p21ras, MAP kinases, PI3 kinase, cdc42/rac, Jak/STAT, NAD(P)H oxidase and others [85].

4.2.5 Oxidative Stress

Oxidative stress has been widely accepted as a key mediator of the initial cellular damage observed in diabetes complications [86]. As a higher content of intracellular glucose is oxidized via the TCA cycle, an increased level of electron donors, NADH and FADH2, enter the electron transport chain and causes uncoupling of the physiological ATP synthesis mechanism in the mitochondria, giving rise to formation of reactive oxygen species (ROS).

ROS are highly unstable products derived from the addition of electrons to oxygen that can readily oxidize proteins, lipids, carbohydrates and DNA leading to cellular structural damage and dysfunction through lipid peroxidation, activation of nuclear factor of Kappa-B (NF-kB), production of peroxynitrite, PKC activation and induction of apoptosis [49, 87]. Brownlee has proposed that oxidative stress is the common upstream pathway to other processes of cellular damage, including the formation of AGEs and the activation of PKC. On the other hand, formation of ROS may be induced by AGEs interacting with RAGE and by products of the PKC pathway activation, perpetuating the oxidative stress state [88].

Free radicals may also be produced by non-mitochondrial sources in the cytosol, and the NOX protein family, the catalytic component of the multiprotein enzyme complex NADPH oxidase, represents the primary non-mitochondrial source of ROS. Increased expression of several of the NOX regulatory subunits, including NOX4, p22phox, p47phox and p67phox have been associated with matrix expansion in animal models [49]. The cell is equipped with a defense mechanism represented by various anti-oxidant enzymes, including the superoxide dismutase to degrade or inactivate the free radicals. However, when the production of ROS exceeds the anti-oxidative mechanisms, a state of oxidative stress is established [79].

The theory that oxidative stress is a primary event leading to the derangement of cellular function in the development of DN has been supported by a body of evidence showing that inhibition of the ROS production attenuates or suppresses cellular damage in DN (86 and others). However, a new theory has been proposed suggesting that increased superoxide formation may be in fact beneficial [89]. This theory is based on a new concept called mitochondrial hormesis, in which the increased formation of superoxide is in fact a compensatory mechanism to restore the reduced levels of superoxide induced by high intracellular glucose. While evidence is cited in defense of the hormesis theory, this issue remains unsettled. A conciliatory concept has been raised by Kumar et al. [90] proposing that both conditions, an initial increase followed by a reduction in superoxide, may exist along the spectrum of mitochondrial oxidative stress.

4.2.6 *Inflammatory Cytokines*

A potential participation of cytokines in the pathogenesis of DN was suggested for the first time in 1991 by Hasegawa et al. [91]. It has been suggested that inflammation in diabetic nephropathy is primarily induced by ROS through activation of the NFkB intracellular signaling pathway [92]. Increased expression of a number of pro-inflammatory cytokines, including IL-1, IL-6, IL-18 and TNF-α in resident and infiltrating glomerular cells have been associated with DN [93–95].

TNF-α directly induces the formation of ROS, and has been associated with hemodynamic alterations, disruption of the filtration barrier, pro-coagulant effects, attraction and adhesion of neutrophils and monocytes to the glomerular area, and induction of apoptosis [94, 95]. IL-1 increases mesangial cell proliferation and synthesis of ECM, contributing to mesangial expansion and thickening of the glomerular basement membrane. This cytokine induces endothelial pro-coagulant activity and increases endothelial permeability [94]. IL-6 increased expression in cells infiltrating the mesangium, interstitium and tubules showed a positive correlation with severity of mesangial expansion. Additional effects of IL-6 observed in DN include altered permeability of glomerular endothelium, increased expression of fibronectin and thickening of the GBM [93, 94]. IL-18 is a potent pro-inflammatory cytokine, which stimulates the production of other inflammatory cytokines including IL-1, IL-6 and TNF-α. In diabetic individuals, renal expression of IL-18 is up-regulated and the serum and urine levels of this cytokine are elevated in comparison with non-diabetic subjects. Most importantly, there is a positive correlation between IL-18 levels and albuminuria [92].

4.2.7 *ER Stress*

The endoplasmic reticulum's (ER) normal function includes a quality control mechanism of protein folding, before the newly synthesized protein is exported to the Golgi apparatus. Misfolded proteins are ubiquitinated and degraded in proteasomes. In diabetes, altered glycosylation, nutrient deprivation, and oxidative stress interfere with the protein folding process, leading to accumulation of misfolded protein and resulting in ER stress. The unfolded protein response is then activated to decrease unfolded protein and increase the folding capacity. This mechanism is mediated by at least 3 transmembrane proteins including inositol requiring enzyme 1 (IRE1), protein-kinase-RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [96]. It has now been recognized that ER stress underlies a growing number of cellular events, especially in mesangial cells and podocytes contributing to development of DN [96, 97].

4.2.8 MicroRNAs

MicroRNAs (miRNAs)are non-coding short RNAs that regulate mRNA expression at the post-transcriptional level, and that play a role in a number of biological processes [98]. Dysregulation of several miRNAs includingmir-192, mir-216a, mir-377, mir-23b, mir-21, mir-93, and mir-29c have been associated with pathological processes in DN [49]. However, miRNAs dysregulation has been reported in cells throughout the whole nephron, participating in a variety of pathological processes affecting the kidney [99, 100].

The precise mechanism by which miRNAs are dysregulated in diabetes and especially in the development of DN is still unclear. Studies of specific miRNAs, their targets and respective altered functions have shed light on several pathological mechanisms related to hyperglycemia, especially in mesangial cells and podocytes [49]. For example, TGF-β, a key factor in fibrosis and inflammation has been reported to up-regulate some miRNAs, including mir-192 and mir-377, thus promoting an increase in collagen and fibronectin expression in mesangial cells exposed to high glucose [98, 101, 102].

In addition to signaling fibrotic processes in kidney diseases, miRNAs may play a role in regulating angiogenesis and glomerular function via vascular endothelial growth factor (VEGF) [100]. Of clinical importance is the fact that circulating miRNA profiles are associated with DN and systemic microvascular damage. These profiles may be useful for risk assessment and may provide an insight into the underlying pathological process during the course of the disease [103].

4.2.9 Podocyte Injury

Podocytes are highly specialized and terminally differentiated epithelial cells with limited or no proliferative capability, under physiological conditions. The morphology of mature podocytes is characterized by a large cellular body with foot processes that attach to integrins in the basement membrane of the glomerular capillaries. Podocytes, together with the glomerular basement membrane and the fenestrated endothelium of the capillaries form the glomerular filtration barrier (GFB), a selective molecular sieve that tightly regulates glomerular filtration. While integrity of all three GFB components is required to maintain a normal glomerular filtration, a podocyte structure named slit diaphragm plays a key role in restricting plasma proteins from leaking into the urine [104, 105]. The slit diaphragm is a 40 nm junction between interdigitating foot processes from neighboring podocytes, and is formed by a complex of proteins including nephrin, podocin, Neph1 and CD2-associated protein (CD2AP). These components are interconnected with intracellular cytoskeletal proteins and play a role in structural changes of podocytes [106].

The podocyte is one of the primary targets of injury in diabetic nephropathy resulting ultimately in deterioration of the glomerular filtration and development of proteinuria. An increased podocyte loss in DN may result from both apoptosis and foot

process effacement with detachment of the podocyte from the capillary wall. Apoptosis is induced by high glucose and may be mediated by TGFβ, Smad7, AGE, angiotensin II and ROS [107, 108]. Foot process effacement results from an active rearrangement of the actin cytoskeleton [109]. Because mature podocytes have a limited capacity to proliferate, apoptosis and foot process effacement will result in decreased number of glomerular podocytes, which correlates with disease progression.

Components of the slit diaphragm, such as podocin and synaptopodin are down-regulated in DN. Changes in nephrin expression however, are yet unclear and may vary according to the stage of the disease [107]. Furthermore, alteration of the slit diaphragm protein complex leads to activation of signaling pathways affecting podocyte function [106]. High glucose levels increase the formation of AGEs, which upon interaction with RAGE elevate ROS production leading to activation of different isoforms of the protein kinase C (PKC) family. Signaling through PKCα appears to affect the development of albuminuria and maintenance of the glomerular filtration barrier structure, while hyperglycemia-induced activation of the PKCβ pathway has been associated with renal fibrosis [110].

Podocytes respond to a number of metabolic and endocrine factors, including angiotensin II, aldosterone, prorenin, insulin, adiponectin, sex hormones, growth hormone, and vitamin D. Since these factors are often altered in diabetes mellitus, they may intensify the direct effect of high glucose on podocyte function derangement [111].

4.2.10 Endothelial Cells

Glomerular endothelial cells are fenestrated, i.e. they possess 50–80 nm pores in the cellular membrane that could allow serum proteins to leak out of the capillary lumen. However, evidence shows that glomerular endothelial cells play an important role in the glomerular filtration barrier. Some studies have demonstrated that damage to endothelial cells may be more closely related to increase in albuminuria than podocyte abnormalities. Nonetheless, the mechanisms linking endothelial cell injury and albuminuria are yet unclear. It has been suggested that loss of the endothelial surface layer (ESL) plays a critical role in protein leaking from glomerular capillaries [112]. The ESL covers the luminal side of capillary walls and is comprised of negatively charged glycoproteins, glycosaminoglycans, and membrane-associated and secreted proteoglycans [113].

Cross talk exchanging information between glomerular cells through different systems appears to play a major role in transmitting the effects of injury from one cell type to another. In this regard, glomerular endothelial cells may interact with podocytes by a bidirectional diffusion of cytokines and growth factors through the basement membrane. Communication between interdigitating mesangial and endothelial cells can also occur through gap junctions. Another mechanism is a paracrine ligand/receptor interaction between endothelial and mesangial cells or endothelial cells and podocytes [112]. Experimental and clinical data in DN have

shown that increased production of cytokines and growth factors by dysregulated glomerular endothelial cells can affect both mesangial cells and podocytes. Conversely, glomerular endothelial cells can be targeted by cytokines and growth factors produced by injured mesangial cells and podocytes [107]. This complex cell-cell interaction contributes to damage of the glomerular filtration barrier leading to albuminuria.

4.3 Diabetic Nephropathy: Potential Treatment

Despite emerging strategies and active investigation, no current single treatment can reverse, or at least stop, renal failure progression in DKD. At best, some of the measures can partially slow the rate at which renal function is lost [114].

The change in albuminuria is often used to assess drug efficacy in human intervention trials in DN [115], but recently the effectiveness of this method has been questioned. More clinical trials are required to establish treatment efficacy in terms of relevant clinical end points reflecting renal function: GFR decline rate or incidence of end-stage renal disease (ESRD) [114].

Current management of DN involves classical nonspecific treatment, including tight glycemic control, hypertension control, as well as weight loss, smoking cessation and anti proteinuric treatments by blockade of the renin angiotensin aldosterone system (RAAS) [114].

In this section we will discuss both classical treatment modalities as well as novel experimental treatment strategies.

4.4 Glycemic Control

The most effective method to prevent diabetic nephropathy is tight glycemic control. The concept of the toxicity of hyperglycemia ("glucotoxicity") has been well accepted as a central mechanism of DKD. Studies in both type 1 and type 2 diabetes have demonstrated the important benefits of intensive glycemic control in reducing the risk of onset of microalbuminuria and progression of renal failure.

The protective benefit of tight glucose control on DN that persists long after the period of tight control (metabolic memory) was reported in trials such as the DCCT-EDIC study [116].

Level of glycemic control to optimize safety is still under debate according to the modified guideline [12]. The recommended target is HbA1c of 7 %, following the recent series of three large clinical trials [36, 117–119] that found no benefit of more intensive glycemic control but showed an increased risk of severe hypoglycemia, when the target is tight glycemic control in the elderly [120].

4.5 Blood Pressure Control

Based on the most recent Joint National Committee (JNC) 8 and KDIGO guidelines, BP levels in diabetes are recommended to be below 140/90 mmHg [121–123].

Hypertension is one of the most common co-morbidities in DKD. Arterial hypertension is an early and frequent phenomenon in any GFR level drop. Blood pressure tends to be higher in diabetic patients with CKD and more common in diabetic than in non-diabetic CKD patients. Approximately two-thirds of all patients with diabetes are hypertensive. Blood pressure control, regardless of the anti-hypertensive agent chosen, can slow the progression of DN. ACEi and ARBs are still recommended as first choice in DKD.

To normalize hypertension in DN, patients need, on average, 3 drugs including diuretics and calcium blockers. Tight blood pressure control, defined as a blood pressure of less than 140/90 mmHg, reduced the risk of death and complications related to diabetes mellitus [4, 9]. However, according to the HOT and ACCORD trials blood pressure treatment targets should not be below 120 mmHg.

4.6 Weight Loss

Obesity is an increasing problem in the general population and in the diabetic population. Several studies indicate that severe obesity, BMI > 40 kg/m^2, enhances the deterioration of renal failure [124]. Weight loss improves glycemic control. Bariatric surgery is suggested as a treatment option for diabetic kidney disease, but although bariatric surgery is associated with reduction in albuminuria and improved glycemic control, its effect on the progression of diabetic kidney disease is not known [125].

4.7 Protein Restriction

Currently, a dietary protein intake of 0.8–1 g/kg body weight per day is recommended by the DKD guidelines [123]. Although animal experiments have demonstrated excellent renoprotective effects of low protein diet, human clinical trials testing the effects of protein restriction on diabetic nephropathy did not show clear benefits of a low protein-restriction diet [126–129].

4.8 Lipid Lowering Drugs

Lipid-lowering agents are indicated for primary and secondary prevention of CVD in diabetes, hypertension and nephropathy, as all increase the CVD morbidity and mortality [12, 130].

The Study of Heart and Renal Protection (SHARP) randomized trial shows that patients with early CKD have lipid abnormalities including increased low-density lipoprotein (LDL) cholesterol levels, increased lipoprotein levels and decreased high-density lipoprotein (HDL) cholesterol levels [131]. In patients with type 1 diabetes mellitus, total and LDL cholesterol levels have been found to be dependent risk factors for the progression of renal disease [132].

Meta-analysis of all lipid lowering trials showed only a trend toward GFR improvement, but results were not clear [133].

4.9 Prevention of AKI

Patients with DKD are more susceptible to acute kidney injury (AKI) than patients without DKD [134]. Much less renal recovery occurs after AKI in patients with DKD, and an event of AKI might cause irreversible kidney damage. Use of RAS antagonists may increase the risk of AKI, independent of other AKI risk factors [135, 136]. In a recent study [44], where 3679 patients with diabetes were followed from January 1999 until December 2008 any AKI episode was a risk factor for progression to CKD stage 4.

4.10 ACEI and ARBs

Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) are known to reduce proteinuria, and treatment with either drug has been the first-line of therapy in the management of diabetic nephropathy for the past 25 years [18]. Blockade of the RAS reduces the incidence of renal events in nephrotic patients with and without diabetes mellitus [137–140], but RAS blockade alone is insufficient in preventing the progression of diabetic kidney disease in many patients [4, 9].

Diabetic animal models have shown that ACE blockers prevent glomerular capillary hypertension with long-term protection against the development of diabetic glomerulopathy, but large human clinical studies have failed to show the same level of protective benefit on human renal function [123]. However, it should be considered that RAS blockade is no longer indicated with rapid or acute elevations of GFR. Most trials of RAS blockade excluded patients with stage 3 CKD and above [123, 141].

While targeting the renin angiotensin system (RAS) shows improvement in the treatment of DN [18, 139, 140], attempts to maximize RAS blockade produced disappointing clinical results [142]. Combined ACEI and ARB therapy was associated with an increased risk of the primary end-point of death from cardiovascular causes in ONTARGET, ALTITUDE, and VA NEPHRON-D clinical trials [143–149]. Current evidence supports avoiding dual renin-angiotensin system blockade in patients with chronic kidney disease.

4.11 Mineralocorticoid Receptor Blockade

Blockade of the renin-angiotensin-aldosterone system (RAAS) is today a standard therapeutic intervention in diabetic patients with chronic kidney disease (CKD). Concomitant mineralocorticoid receptor blockade has been shown to further slowdown CKD progression [150]. Combination of spironolactone and ARB or ACEi improved BP control and decreased proteinuria, but also increased dramatically the prevalence of hyperkalemia [151]. Clinical use of mineralocorticoid receptor blockade should be extremely cautious, with frequent reassessment of K^+ levels.

The effect of finerenone, a third-generation mineralocorticoid receptor antagonist, was recently studied in DKD in the ARTS-DN clinical study (n = 821 DN patients in the study group). Finerenone combined with ACE or ARB shows reduction in proteinuria, but with no effect on GFR loss or new hyperkalemia events [152].

4.12 New Therapies for Diabetic Kidney Disease

Despite the availability of renin-angiotensin system (RAS) blockers, the continued increase in diabetic ESRD urgently requires additional new disease modifying therapies.

With extensive research in the field, there is a growing area for the development of potential new therapies, targeting fibrosis, oxidative stress, inflammation and other metabolic signaling pathways that are known to take part in the pathogenesis of DKD. In recent decades, most large human clinical trials have failed to show prevention of ESRD. We will summarize the important human clinical trials studying new approaches for treating DKD.

Oxidative stress and inflammation have been increasingly described to be active in the pathogenesis of CKD, in particular in DKD. Therefore, the Bardoxolone clinical study raised many expectations for new antioxidant therapy to improve advanced CKD. Bardoxolone methyl is a synthetic triterpenoid that activates the Nrf2 pathway, restoring antioxidant response. Although early human studies with T2DM and CKD stage 3–4 reported reductions in serum creatinine concentration [153], the BEACON trial was stopped after 9 months, when 43 patients receiving bardoxolone methyl developed ESKD and increased cardiovascular morbidity and mortality [154, 155].

Advanced Glycation End Products (AGE) have been implicated in playing a major role in the progression of microvascular and macrovascular diabetic complications. Blockage of AGE formation or anti RAGE were attractive treatment strategies [156], but so far studies with nonspecific blockers of AGE formation [157, 158] and also anti RAGE have been found unsafe in human clinical trials. Thus, until safer drugs are developed, the only alternative for patients with DKD should be a low AGE diet [156].

Pyridoxamine (vitamin B6) has been shown in animal studies to be a natural inhibitor of AGE that reduces albuminuria and preserves renal function [159], but small randomized double-blind placebo-controlled trials in T2DM patients conducted by Lewis et al. have shown negative results so far [160, 161].

Vitamin D treatment has been shown in animal studies to reduce albuminuria, podocyte effacement and increased glomerular basement membrane [162]. Vitamin D levels are low in the serum of diabetic patients and replacement therapy with an active analogue was aimed to reduce proteinuria, interstitial fibrosis and improve renal function. One meta-analysis of vitamin D analogues in humans has shown a reduction of proteinuria in combination with RAAS blockade using an ACEi/ARB [163], but a recent meta-analysis has shown no significant effect [164].

Adrenocorticotropic hormone (ACTH) reduces proteinuria in non-diabetic glomerulopathy through activation of melanocortin receptors (MCR) expressed in the podocyte. A small pilot study showed that subcutaneous injection of ACTH gel stabilizes renal function and reduces urinary protein for up to 6 months after treatment [165].

Fibrosis is a main common pathway in the progression of DKD and a main target of research in the prevention of renal failure. Pirfenidone is an oral anti-fibrotic agent that inhibits TGF-β in animal models. The effect of pirfenidone was studied in a small advanced human diabetic nephropathy trial that showed an improvement in GFR at 1 year, but gastrointestinal symptoms and fatigue were common adverse symptoms [166].

Endothelin Receptor Antagonists (ET-1) showed promising results in diabetic animal models. The ASCEND trial was a multinational double-blind trial which randomized endothelin antagonist avosentan or placebo with RAS-inhibition. Although proteinuria was reduced, the trial was stopped due to an excess of congestive heart failure secondary to hypervolemia [167]. Daglutril, a combined endothelin-converting enzyme and neutral endopeptidase inhibitor, safely improved blood pressure control, but did not affect proteinuria in patients with diabetic nephropathy [168].

The relevance of inflammation in the pathogenesis of DKD has been investigated in recent years [169]. Pentoxifylline is a methylxanthine derivative with inhibitory actions against TNF-α. The PREDIAN trial shows significant reductions in proteinuria and preservation of renal function (eGFR decreased by 2.1 ± 0.4 mL/min per 1.73 m^2 in the Pentoxifylline group ($n = 82$) compared with 6.5 ± 0.4 mL/min per 1.73 m^2 in the control ACEi/ARB in CKD stages 3/4 and T2DM [170]). The levels of TNF-α, a central cytokine in the mechanism of DKD in this study, were decreased in the urine of the treated group.

Antioxidant therapies are a potential target. Recently, the result of a study on probucol (a lipid lowering drug that decreases oxidative stress and NOX2) combined with telmisartan showed improved reduction in proteinuria in the probucol and telmisartan group [171].

Experimental and clinical studies have suggested that uric acid may contribute to the development of DKD. Allopurinol reduced albuminuria in 4 months of treatment in 40 patients with early DN. More recently, the PERL trial has begun recruiting patients with T1DM and CKD stages 1–3 to be given allopurinol for a 3-year period. Results of this trial are still pending. Allopurinol has also recently been reported in T2DM to significantly decrease serum uric acid and proteinuria, and improve eGFR compared to conventional treatment [172–174].

There are clear and urgent needs to improve our treatment arsenal for DKD. New interventional therapeutic targets should come from basic molecular research using

new and more effective biomarkers. Early identification of patients at risk for incidence or progression to end-stage renal failure and early treatment are highly desirable. Meanwhile, we should focus on improving glycemic control without episodes of hypoglycemia and on achieving better blood pressure control.

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Chapter 5
Immune-Mediated Mechanisms of Proteinuria

Lindsey Goetz and Joshua M. Thurman

Abbreviations

APC	Antigen presenting cells
AT1R	Angiotensin II Type I Receptor
C3G	C3 glomerulopathy
CNI	Calcineurin inhibitors
CTLA-4	Cytotoxic T-Lymphocyte–Associated Antigen 4
FcR	Immunoglobulin receptor
FSGS	Focal segmental glomerulosclerosis
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
IC	Immune-complex
LPS	Lipopolysaccharide
MBL	Mannose binding lectin
MCD	Minimal change disease
MN	Membranous nephropathy
MPGN	Membranoproliferative glomerulonephritis
PLA2R1	M-type Phospholipase A2 Receptor 1
THSD7A	Thrombospondin Type-1 Domain-Containing 7A
TLR	Toll-like receptor

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5.1 Introduction

The kidney is a common target of autoimmune and inflammatory diseases. Some of these diseases affect multiple tissues throughout the body, but many inflammatory diseases specifically target the kidney. The susceptibility of the kidney to inflammatory injury may reflect the distinctive structure of the glomerulus. The kidney receives a large portion of the cardiac output (~20%), and blood cells and plasma proteins are concentrated within the glomerulus due to the filtration out of water. The ultrastructure of the glomerulus as water is filtered out. The fenestrated endothelium exposes the underlying basement membrane. The glomerular basement membrane (GBM) is negatively charged, which provides a barrier to passage of negatively charged proteins, such as albumin, but facilitates the deposition of positively charged proteins such as some IgG molecules. Thus, the structure of the glomerulus likely contributes to its frequent involvement in inflammatory diseases, as well as to its susceptibility to inflammatory injury.

It is worth noting that many inflammatory cells and molecules can have a strong pathologic effect on target tissues without causing overt inflammatory changes on light microscopy. Examination of the role of the immune system in the pathogenesis of the nephrotic syndrome requires careful experimentation, and cannot be based simply on the detection of classical "inflammatory" changes by conventional histology. Experiments in which specific immune molecules or cells are targeted by genetic manipulation or by therapeutic drugs have revealed unexpected roles for the immune system in disease. The clinical use of drugs that target parts of the immune system have also improved our understanding of the diseases that cause the nephrotic syndrome. Rituximab, for example, has been used effectively to treat diseases in which a pathogenic role for B cells was not previously well established. The efficacy of immunomodulatory drugs indicates that the immune system is likely involved in the pathogenesis of diseases such as minimal change disease (MCD), even though light microscopy does not reveal infiltration of the glomerulus by inflammatory cells.

5.2 The Glomerular Capillary Wall

The glomerular capillary wall is comprised of a fenestrated endothelium, the GBM, and podocytes. This structure allows the free passage of water and small molecules, but passage of larger proteins, such as complement proteins and immunoglobulin, is restricted. By definition, proteinuria indicates a disruption of this protein selectivity.

A distinction is often made between the nephrotic syndrome and the nephritic syndrome. The nephritic syndrome manifests with proteinuria (frequently subnephrotic), hematuria and/or pyuria, and a reduction in the glomerular filtration rate (GFR). The diseases that cause the nephritic syndrome are often autoimmune or inflammatory in nature, and inflammation (e.g. infiltration of polymorphonuclear neutrophils, macrophages, and lymphocytes) is seen within the glomeruli by light microscopy. The diseases associated with the nephritic syndrome typically cause

damage to and/or immune-complex (IC) deposition within the mesangium, glomerular endothelial cells, or the GBM. The development of overt glomerular inflammation in this context is likely due to the entry of chemotactic factors (C5a and chemokines) into the circulation and exposure of adhesion molecules on glomerular endothelial cells to leukocytes.

Immune-mediated injury that is limited to podocytes, in contrast, is more likely to cause the nephrotic syndrome. The nephrotic syndrome is generally associated with a greater degree of proteinuria (>4 g/day) than the nephritic syndrome. Hematuria and pyuria are less prominent than the nephritic syndrome, and the GFR is initially better preserved. Podocyte injury in these diseases is generally not associated with significant glomerular inflammation by light microscopy, likely because chemotactic factors, such as C5a, pass into the urine instead of into the bloodstream (Fig. 5.1).

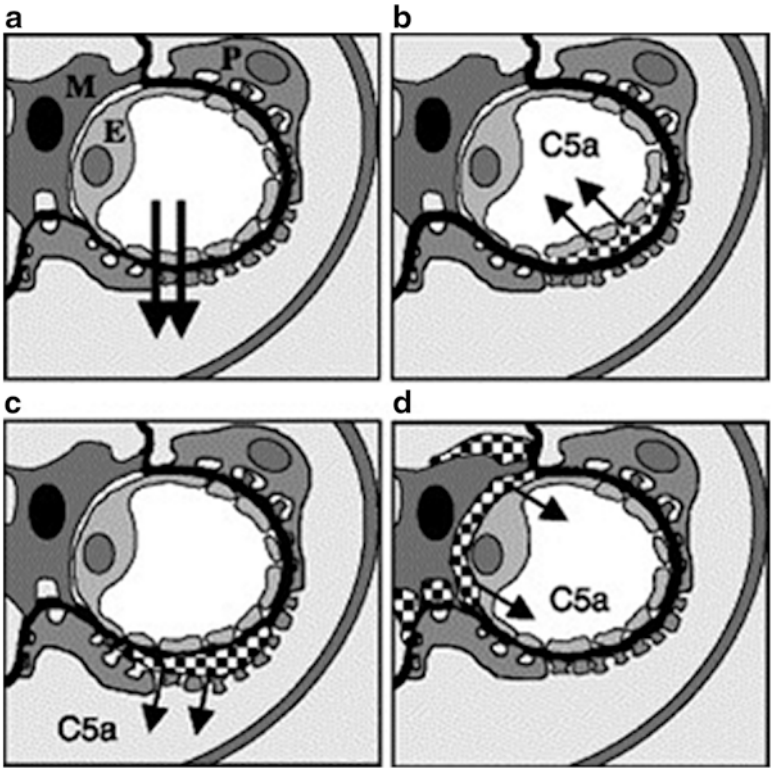

Fig. 5.1 Immune-complexes and glomerular inflammation. (**a**) In the healthy kidney, fluid and solute pass across the glomerular capillary wall into the urinary space. (**b**) Subendothelial immune-complexes (checkered area) generate chemotactic factors, such as C5a. Because of the location of the immune-complexes the C5a can enter the circulation and recruit inflammatory cells. (**c**) C5a generated by subepithelial immune-complexes enters the urinary space. Because less of the C5a enters the circulation there is less recruitment of inflammatory cells to the glomerulus. (**d**) C5a generated by mesangial immune-complexes can enter the circulation and recruit inflammatory cells. Reproduced with permission from: L.A Trouw, M.A Seelen, and M.R Daha. Complement and renal disease. Molecular Immunology, 2003;40(2–4):125–34

There is a large degree of overlap in the causes of the nephritic and the nephrotic syndromes. For example, IgA nephropathy and membranoproliferative glomerulonephritis (MPGN) are characterized by mesangial and subendothelial IC deposits, and glomerular inflammation is seen on light microscopy. Patients with these diseases usually present with the nephritic syndrome, but in some cases patients with these diseases present with the nephrotic syndrome, suggesting greater damage to the podocytes than to the mesangial and endothelial compartments. It is not surprising that there is overlap in the glomerular disease syndromes as there is cross-talk between podocytes, glomerular endothelial cells, and mesangial cells [1]. Consequently, activation or injury to any glomerular structure also affects the other glomerular cell types. Furthermore, even though the podocytes play a critical role in the filtration barrier, isolated damage to glomerular endothelial cells or the GBM can also cause proteinuria, even with apparently normal podocytes. For example, selective experimental injury of glomerular endothelial cells or the GBM causes proteinuria without podocyte foot process effacement [2].

5.3 Immunologic Molecules That Alter Glomerular Protein Trafficking

It is artificial to speak about specific cells or molecules of the immune system in isolation as they operate as part of an orchestrated inflammatory response. Nevertheless, many studies have revealed important functional roles for specific molecules within the immune system. There is also an expanding array of drugs that target specific molecules of the immune system, so it is important to identify the pathways and molecules that contribute to disease pathogenesis.

5.3.1 Soluble Permeability Factors

Clinical observations suggest that soluble "permeability factors" cause proteinuria in some patients with the nephrotic syndrome (Fig. 5.2). Approximately 30–50 % of patients with focal segmental glomerulosclerosis (FSGS) have recurrence of their disease after renal transplantation. Recurrence usually occurs within 1 year, and sometimes within hours after transplantation. FSGS is also frequently responsive to plasmapheresis and immunoadsorption [3], treatments that presumably work by removing plasma components. Furthermore, serum isolated from patients with recurrent FSGS increases the permeability of the glomerular filtration barrier to albumin in vitro and in animal models in vivo [4, 5]. Many putative candidates, both immunologic and non-immunologic, have been identified over the past two decades. Despite intensive study, however, the molecular identity of the permeability factor remains elusive.

Adsorption of plasma proteins using Protein A, a component of the Staphylococcus Aureus bacterial cell wall with natural affinity for mammalian proteins, particularly immunoglobulins, reduces proteinuria and isolates a factor that increases glomerular

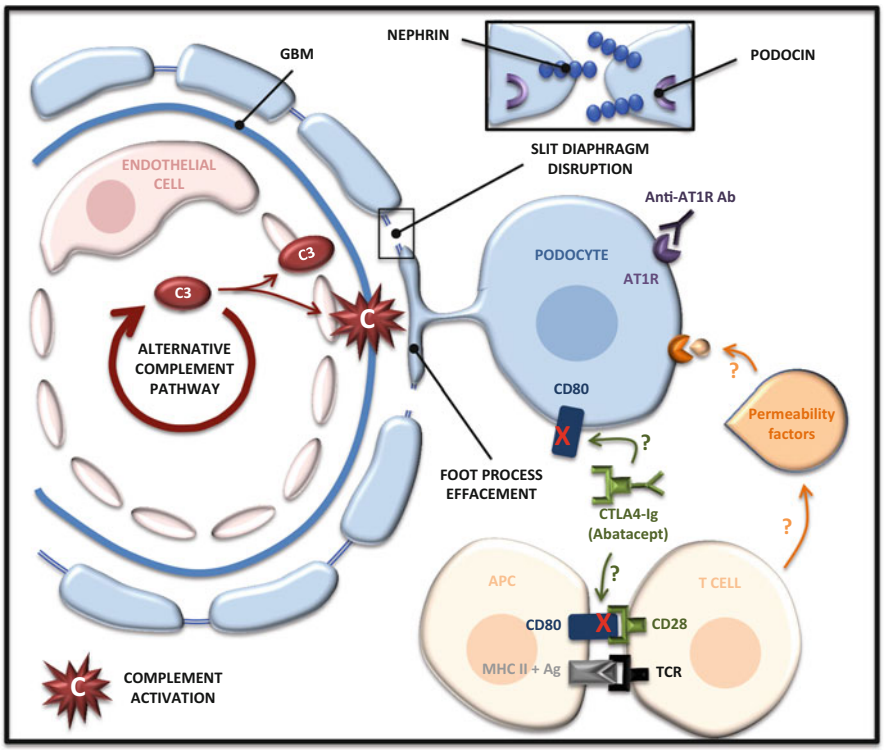

Fig. 5.2 Soluble factors that may contribute to glomerular injury. Uncontrolled alternative complement activation generates soluble fragments with biological activity. Other soluble permeability factors have been hypothesized to contribute to podocyte injury with resultant foot process effacement and slit diaphragm disruption. Abatacept may mitigate glomerular injury by disrupting CD80-dependent signaling in podocytes and/or leukocytes. AT1R = Angiotensin II Type 1 Receptor; TCR = T cell receptor; APC = antigen presenting cell; MHC = major histocompatibility complex; Ab = antibody; Ag = antigen

permeability [3]. Early studies characterized the permeability factor as a 30–50 kD hydrophobic protein [5], too small to be an immunoglobulin, although other studies have induced proteinuria in animals through the transfer of immunoglobulin purified from patients with the nephrotic syndrome [6]. Autoantibodies to the Angiotensin II Type I Receptor (AT1R), expressed by podocytes in addition to vascular endothelial cells, have been implicated as possible permeability factors in some patients with FSGS [7]. Interestingly, this mechanism of proteinuria may not be specific to FSGS, as anti-AT1R antibodies have also been associated with the development of proteinuria and hypertension in pre-eclampsia [8]. Antigen arrays have identified several autoantibodies that correlate with FSGS recurrence after transplantation [9], further suggesting a role for autoantibodies as pathologic factors in this disease. The strongest predictor of disease recurrence was seen with antibodies that recognize CD40, a T cell co-stimulatory protein. CD40 expression was seen in biopsies from patients with FSGS, but not in control biopsies. Immunologic proteins other than immunoglobulins

have also generated interest as putative circulating permeability factors in recurrent FSGS, including CLCF-1, a 22 kD B cell-stimulating cytokine in the IL-6 family [10].

5.3.2 *Antibody-Mediated Glomerular Disease*

For more than 40 years, it has been recognized that immunostaining of kidney biopsies from some patients with nephritic and the nephrotic syndromes reveals glomerular deposits of immunoglobulin and complement proteins [11]. Glomerular antibody deposition is seen in patients with underlying autoimmune diseases, infectious diseases, and paraprotein-related disorders. The term "immune-complex" refers to an antibody bound to a specific target antigen. ICs can be composed of a single antibody bound to a single antigen. Because immunoglobulin is multivalent, however, it can cross-link multiple antigens and form larger complexes.

A longstanding paradigm has been that glomerular ICs can result not only from the deposition of circulating ICs, but also from antibodies that bind to intrinsic glomerular antigens or to antigens that lodge within the glomerular capillary wall (Fig. 5.3). The location of the ICs within the glomerulus likely determines the clinical manifestations. For example, subendothelial ICs cause endocapillary proliferation and the nephritic syndrome, whereas subepithelial ICs cause podocyte damage and the nephrotic

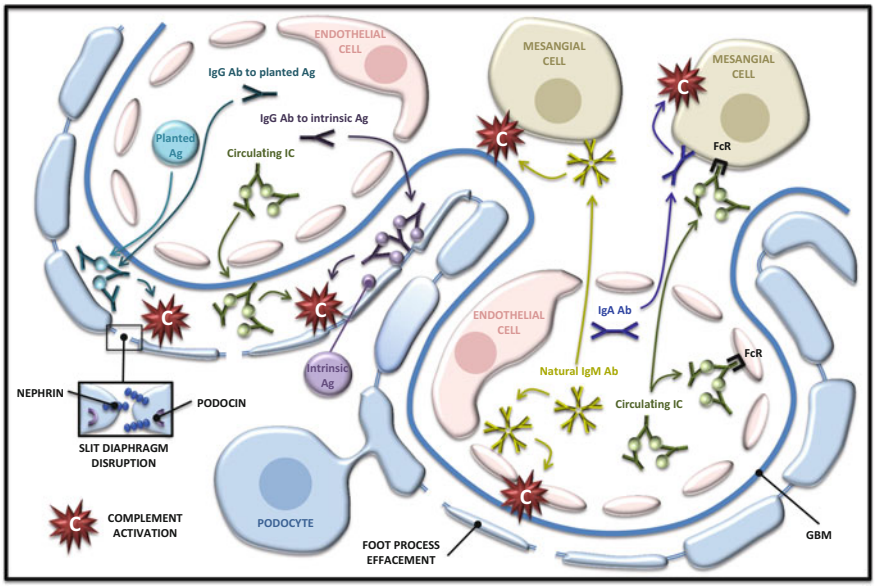

Fig. 5.3 Antibody-mediated glomerular injury occurs through multiple mechanisms. Antibody may bind directly to either intrinsic, glomerular antigens or planted antigens from other locations with subsequent immune-complex formation. Preformed, circulating immune-complexes also may deposit in the glomerular capillary wall. These immune-complexes can then activate complement or engage Fc receptors to cause glomerular injury and proteinuria. Ag = antigen; Ab = antibody; IC = immune-complex; FcR = Fc receptor

syndrome. As an example of this, subendothelial ICs in patients with lupus nephritis are associated with class III or IV disease (proliferative), whereas subepithelial ICs are associated with class V disease (membranous). The location of the ICs within the glomerular capillary wall is influenced by the identity of the antigen as well as by the size and charge of the ICs. Small ICs can pass more easily through the glomerular capillary wall and are more likely to deposit within the subepithelial space.

The Complement System Once deposited, ICs can trigger an inflammatory response through several pathways. The complement protein C1q binds to IgG and activates the classical pathway of complement. The affinity of C1q for a single IgG molecule is low. C1q has six IgG binding regions, however, so the affinity increases dramatically when multiple IgG molecules are clustered in one region. IgM is an even stronger activator of complement than IgG, likely because it exists as a pentamer or hexamer, so there are multiple binding sites for C1q on a single IgM. The ability of IgG to activate complement also depends on the isotype. IgG3 is the strongest activator of complement, and IgG4 is the weakest. Interestingly, antibodies to the M-type Phospholipase A2 Receptor 1 (PLA2R1) in membranous nephropathy (MN) are usually IgG4 (non-complement activating), yet C3 fragments are deposited in the glomeruli of most affected patients [12]. One possible explanation for this discrepancy is that IgG4 may activate the mannose binding lectin (MBL) pathway of complement, although a pathogenic role for the MBL pathway in MN has not yet been established. Nevertheless, a large body of experimental data suggests that complement activation is an important cause of glomerular injury in membranous disease.

IgM and complement proteins are also seen in the glomeruli of patients with MCD and FSGS, diseases not traditionally regarded as "immune-complex mediated" due to the lack of inflammatory changes by light microscopy. The detection of IgM and C3 in these diseases has been attributed to the passive trapping of these large molecules in scarred regions of the glomeruli. Recent work has shown, however, that IgM binds to neo-epitopes expressed in injured glomeruli and activates the complement cascade [13, 14]. Strategies that prevent glomerular IgM deposition and complement activation reduce proteinuria in several experimental models. This inflammatory response to tissue injury may, therefore, be a common final pathway of disease progression in patients with various forms of glomerulopathy.

Fc Receptors ICs also trigger an inflammatory response through their interaction with receptors for the constant region of immunoglobulin (Fc receptors, or FcRs). FcRs are expressed on lymphocytes, myeloid cells, and dendritic cells. Several Fc receptors activate an inflammatory response to ICs (FcRI and FcRIIIa) through intracellular signals, and one of the Fc receptors inhibits the inflammatory response (FcRIIb) [15]. The Fc receptors are important both in generating an immune response against antigen and in maintaining tolerance. They also trigger the downstream effector pathways that mediate IC-induced inflammation. Binding of IgG to the FcRs can elicit cell activation or inhibition pathways, promote cytokine production, and influence IC clearance and trafficking.

Several studies have shown that signaling through the Fc receptor is an important cause of IC-mediated glomerular injury. In a mouse model of lupus nephritis, targeted

deletion of the gene for the Fc receptor protected the mice from glomerular injury, even though glomerular complement activation was still detected [16]. In another model of lupus-like disease, however, deletion of the gene for the Fc receptor was not protective [17]. Therefore, the role of FcRs in inflammatory glomerular disease is probably influenced by other inflammatory signals. The efficacy of IVIg for ameliorating autoimmune diseases is likely mediated, in part, through the interaction of the administered immunoglobulin with FcRs [18], and several therapeutic agents that specifically modulate inflammation by targeting FcR signaling are in development.

Membranous Nephropathy MN is the prototypical example of IC-mediated the nephrotic syndrome. MN has been extensively studied such that the pathophysiology of this disease is now very well understood. Animal models of MN have been developed that accurately replicate the histologic and ultrastructural changes seen in human disease. Studies of animal models and of patients with MN have provided important insights into the pathogenesis of IC-mediated the nephrotic syndrome.

In MN, ICs are detected in the subepithelial space, resulting in podocyte injury and the nephrotic syndrome. Because subepithelial ICs are so prominent in MN, studies have focused on identifying the target antigens and on determining whether they are podocyte antigens (intrinsic) or non-podocyte antigens (planted). Many different target antigens have now been identified. In several cases of neonatal MN, it was discovered that the mothers had developed antibodies to neutral endopeptidase [19]. Transplacental passage of the antibodies caused MN in the newborns, but the disease spontaneously remitted as the titer of maternal antibody in the babies diminished. Although only four such cases have been reported, they provide important insight into the pathogenesis of MN.

More recently, PLA2R1 was identified as a target antigen in the majority of cases of primary MN [20] with several studies since confirming this finding. PLA2R1 is expressed on podocytes, and antibodies to this protein are detectable in the serum of approximately 70 % of patients with idiopathic disease. The protein Thrombospondin Type-1 Domain-Containing 7A (THSD7A) was also identified as a target antigen in a subset of MN patients [21]. Interestingly, antibodies to THSD7A were not detected in patients with antibodies to PLA2R1, suggesting that autoantibodies to each of these two podocyte proteins occur in distinct groups of patients.

Several planted antigens have also been identified in patients with MN. Bovine serum albumin (a protein derived from dietary cow's milk) serves as an antigen in some cases of childhood MN [22]. Approximately 20 % of MN cases are associated with systemic illnesses such as autoimmune diseases, infections, or cancer [19]. In these "secondary" forms of MN, disease-related antigens may deposit in the glomerular capillary wall. In patients with systemic lupus erythematosus, for example, DNA and other self-antigens have been identified in the glomerular immune deposits. In patients with chronic infectious diseases, such as hepatitis C, infection-associated antigens have been identified within the glomeruli.

When MN is caused by the formation of antibodies to self-proteins (either intrinsic to the podocyte or planted antigens), it involves a loss of tolerance to self and can be considered an autoimmune disease. Primary MN has been associated with

polymorphisms in the gene for PLA2R1 [23], although it is not clear why genetic variation in PLA2R1 predisposes patients to an autoimmune response against the protein. When the immune response is directed against a foreign, planted antigen, such as an infection-associated antigen, the formation of antibodies is appropriate and thus does not constitute autoimmunity. However, deposition of the ICs within the glomerular capillary wall leads to pathologic inflammatory injury of the podocyte and the filtration barrier. Treatment with immunosuppressive drugs may be equally effective in MN caused by autoantibodies to self-proteins and by antibodies to foreign, planted antigens. However, immunosuppression can be detrimental in patients with underlying infections or cancers and must be considered carefully in this context.

5.3.3 Complement-Mediated Glomerular Disease

As discussed above, the complement cascade is an important mediator of injury caused by glomerular ICs. Another category of glomerular disease that has recently been described is C3 glomerulopathy (C3G), a disease in which uncontrolled activation of the alternative pathway of complement causes glomerular injury in the absence of ICs [24]. Most patients with C3G develop the nephritic syndrome and a MPGN pattern of injury by light microscopy, but approximately 15 % of patients present with the nephrotic syndrome [25] in which case the glomeruli may appear normal by light microscopy [26]. Glomerulosclerosis is a common development in progressive disease.

C3G is usually associated with congenital or acquired defects in the proteins that control activation of the alternative pathway of complement [27]. The defects in complement regulation that have been identified in patients with C3G are typically systemic (e.g. genetic mutations or circulating autoantibodies), and it is unclear why the glomerulus is the target of complement-mediated injury in these patients.

5.3.4 Toll-Like Receptors

Toll-like receptors (TLRs) are a family of 10 trans-membrane proteins that are important sensors of infections and/or of tissue injury. The TLRs recognize common microbial motifs as well as “danger signals” that are released by injured cells. Activation of these receptors initiates an intracellular signaling cascade that triggers an inflammatory response. TLRs are primarily expressed by antigen presenting cells (APCs). However, some TLRs, including TLRs 2–4, are also expressed by podocytes and other resident kidney cells and have been implicated in the pathogenesis of proteinuria (Fig. 5.4).

Stimulation of murine podocytes with endotoxin (lipopolysaccharide, or LPS), the ligand for TLR4, increases foot process motility in vitro and induces foot process effacement with proteinuria in vivo [28]. Human podocytes also express TLR3

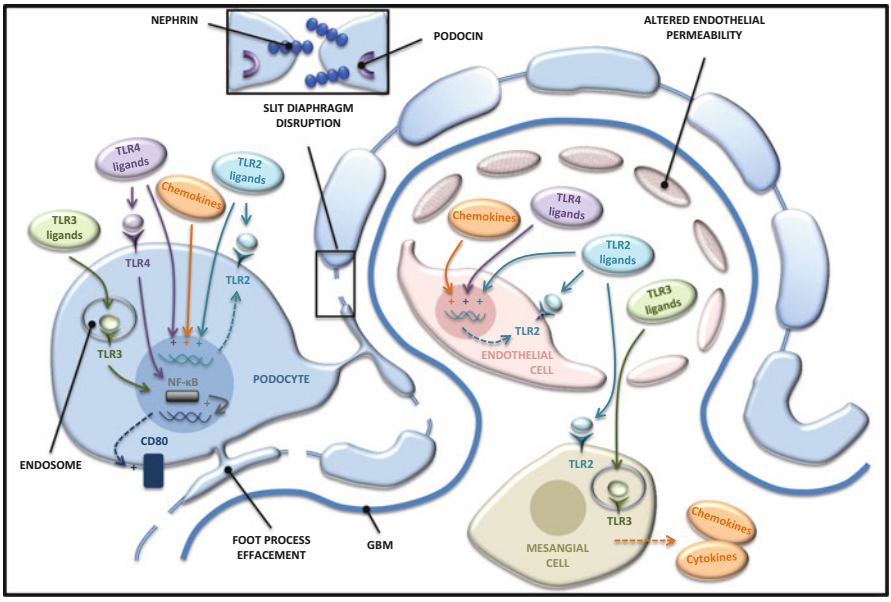

Fig. 5.4 Toll-like receptor signaling in resident glomerular cells leads to glomerular injury. Ligation of constitutively-expressed and inducible toll-like receptors mediates altered podocyte morphology through both CD80 dependent and independent mechanisms. Toll-like receptor signaling also occurs in endothelial cells, which can alter endothelial permeability, and in mesangial cells, which may contribute to glomerular inflammation. TLR = toll-like receptor; Solid arrow = target of action; Hashed arrow = synthesized protein

and develop a similar injury phenotype when exposed to dsRNA, a TLR3 ligand [29]. Furthermore, mice injected with polyIC (a dsRNA analogue) develop nephrotic-range proteinuria with findings on light microscopy reminiscent of MCD. This may help to explain the clinical observation that viral infections sometimes precede the development of proteinuria (e.g. MCD relapse) and implicate local TLR signaling as a contributor to this process.

Podocytes can also express TLR2. However, unlike TLR3 and TLR4, TLR2 expression does not appear to be constitutive, but rather contingent upon an inflammatory microenvironment. Inflammatory cytokines such as TNF-α and IFN-β have been shown to induce podocyte TLR2 expression, for example, as have infection-associated molecules such as LPS and the TLR2 ligand lipopeptide (a component of some bacterial cell walls). Nephritic mice exposed to lipopeptide demonstrate a decrease in and redistribution of nephrin, a key component of the slit-diaphragm, resulting in the transient development of nephrotic-range proteinuria [30]. However, lipopeptide does not induce proteinuria in healthy mice, presumably because inflammation is required to induce sufficient TLR2 expression to mediate the lipopeptide signal. This mechanism may help to explain the clinical observation that bacterial infection sometimes exacerbates proteinuric diseases such as lupus nephritis, but also suggests that isolated TLR2 signaling is unlikely to account for the emergence of de novo proteinuria.

Resident kidney cells other than podocytes also express TLRs that, when activated, may directly and/or indirectly contribute to the development of proteinuria. Murine mesangial cells express TLR2 and TLR3 [30, 31]. These cells respond to stimulation by the appropriate TLR ligands in culture by secreting chemokines and pro-inflammatory cytokines such as IL-6 that contribute to local tissue injury and conceivably cause or exacerbate glomerular disease. Like podocytes, cultured murine endothelial cells also express TLR2 in response to TNF-α, IFN-β, and LPS [32]. When subsequently exposed to lipopeptide, the endothelial cells express injury markers and demonstrate increased permeability to albumin.

5.3.5 CD80 Ligands

B7-1 (CD80) is a co-stimulatory protein that binds to CD28 and Cytotoxic T-Lymphocyte–Associated Antigen 4 (CTLA-4) on T cells. B7-1–CD28 signaling provides a strong second signal for T cell activation, whereas B7-1–CTLA-4 signaling inhibits T cell activation. Although the function of B7-1 was initially studied on antigen presenting cells, B7-1 has also been detected on other cell types, including podocytes. Podocytes increase their expression of B7-1 in response to activation of TLR3 and TLR4 [28, 29]. Further, the podocyte injury mediated by these TLRs appears to be contingent upon the expression of B7-1, and the absence of this protein prevents downstream signaling within the podocyte [29]. It is not clear whether or not podocyte B7-1 plays a functional role in the adaptive immune response, but B7-1 on the surface of podocytes appears to directly cause rearrangement of the podocyte cytoskeleton, independent of T cells and other components of the immune system [28].

Increased podocyte B7-1 expression has been noted in renal biopsies of patients with various forms of the nephrotic syndrome [28, 33]. Further, urinary B7-1 levels have been found to be elevated in MCD [34]. CTLA-4-Ig (Abatacept) is a soluble fusion protein in which CTLA-4 is linked to the Fc portion of IgG1. CTLA-4-Ig blocks the B7-1–CD28 interaction, thereby preventing co-stimulation of T cells. Five patients with FSGS in whom podocyte B7-1 expression was detected in the renal biopsy were treated with CTLA-4-Ig, and all showed a reduction in proteinuria [33]. This study also suggested that CTLA-4-Ig directly blocks the effects of B7-1 on podocyte motility. Another trial examined the efficacy of CTLA-4-Ig in patients with lupus nephritis [31]. The investigators hypothesized that CTLA-4-Ig would block T cell co-stimulation and also directly stabilize podocytes. However, treatment with CTLA-4-Ig was not associated with either an improved response rate or a reduction in proteinuria in this study. However, the patients in this trial were treated with standard immunosuppressive drugs (cyclophosphamide, corticosteroids, and azathioprine), possibly obscuring the effects of CTLA-4-Ig.

5.3.6 Other Cytokines

A large number of biologic agents have been developed as therapies for autoimmune diseases and cancer. Treatment of patients with these drugs has revealed that they can cause proteinuria and glomerular injury. For example, treatment of patients with each form of interferon (IFN-α, -β, and -γ) has been linked with the development of MCD and/or FSGS [32, 35]. These cytokines affect TLR expression on glomerular cells and also affect other immune pathways. Another possible mechanism linking the interferons with glomerular injury is through the induction of APOL1 production [36]. Nevertheless, the mechanisms by which these agents cause glomerular injury are not yet known, and the underlying systemic diseases for which the agents are used may confound the association of these drugs with the development of proteinuria.

5.4 Cellular Immunity and the Nephrotic Syndrome

5.4.1 T Cells

T cells are an important part of the adaptive immune system. A role for T cells in the nephrotic syndrome has been suspected since at least 1974, when Shalhoub hypothesized that a factor released by T cells is the cause of MCD. MCD is distinguished histologically from other patterns of glomerular injury by the conspicuous absence of complement and immunoglobulin deposition, yet it behaves clinically as an immune-mediated process. MCD frequently responds to corticosteroids and cyclophosphamide, for example, and has been shown to remit with measles infections, presumably due to viral suppression of cell-mediated immunity.

Drawing on the additional observations that MCD can occur in patients with Hodgkin's lymphoma and that patients with this pattern of glomerular injury are particularly susceptible to pneumococcal infections, Shalhoub proposed that MCD could be a systemic disorder of T cell function. He further hypothesized that a T cell product alters glomerular permeability either by exerting a direct effect on podocytes or the glomerular basement membrane, or by acting through mesangial cells.

In the decades since, our understanding of T cell biology has progressed considerably. Many new tools are now available to study the different T cell subsets and the soluble factors they produce, and there is additional evidence that T cells can directly affect podocyte function. T cell activation precedes the development of the nephrotic syndrome in a rat model [37]. Moreover, transcriptional analysis of T cells isolated from patients with relapsing the nephrotic syndrome reveals different expression patterns in cells isolated during remission and during disease flares [38]. More recently, NOD/SCID mice were reconstituted with CD34+ stem cells. Mice that received cells from patients with the nephrotic syndrome developed MCD-like disease, whereas mice that received cells isolated from control subjects did not [39]. The authors concluded that immature T cells (reconstituted by the stem cells) rather than mature, circulating T cells mediate the disease.

Evidence is also accumulating to support the contribution of a secreted T cell factor to the development of MCD. Isolates from T cell hydridomas generated from patients with MCD induce proteinuria in rats, with kidney sections taken from these animals revealing evidence of foot process effacement [40]. Podocytes express receptors for T cell cytokines such as IL-4 and IL-13 [41], and rats transgenically manipulated to overexpress IL-13 develop foot process effacement and the nephrotic syndrome [42]. In spite of much progress, however, the definitive identity of Shaloub's soluble T cell factor is yet to be established, and a causal role of T cells in development of MCD and FSGS remains unproven.

T cells may also contribute directly to the development of proteinuria in other forms of the nephrotic syndrome. In Heymann nephritis, a rat model of MN, cytotoxic (CD8+) T cells and their effector molecules (e.g. perforin and granzymes) have been isolated from glomeruli [43]. Furthermore, rats permanently depleted of CD8+ T cells do not develop proteinuria even though C3 and IgG deposition are still seen, providing evidence for a direct contribution of CD8+ T cell cytotoxicity to podocyte injury in this model.

5.4.2 B Cells

Several therapeutic agents that specifically target B cells have been developed, and there is accumulating evidence that these drugs are effective for treating proteinuric renal diseases. Rituximab is a monoclonal antibody to CD20 that depletes B cells but does not deplete plasma cells (the primary producers of immunoglobulin). Several studies have shown that rituximab is effective for the treatment of MN [44, 45]. This may be due to reduced production of nephritogenic antibodies, and treatment with rituximab reduces the levels of anti-PLA2R1 antibodies in the blood [46]. Because most IgG is produced by plasma cells, however, it is possible that rituximab ameliorates MN by interfering with other B cell functions. In addition to production of immunoglobulin, for example, B cells modulate effector T cell differentiation, produce cytokines, serve as antigen presenting cells, provide costimulatory signals to T cells, and orchestrate the formation of lymphoid structures within non-lymphoid organs including the kidney [47, 48]. Thus, even in IC-mediated diseases, the therapeutic effect of B cell targeted therapies may extend beyond a reduction in pathogenic antibody production alone to modulate other aspects of the adaptive immune response.

There are also a large number of case series and clinical trials demonstrating that rituximab may be an effective treatment for MCD and FSGS [49–52], further supporting an immunologic basis for these diseases and indicating an important role for B cells in particular. Although the means through which B cell depletion leads to a reduction in proteinuria remains to be determined, the frequent absence of glomerular IgG deposition in MCD and FSGS suggests that B cells may also contribute to the immunopathology of these diseases in part through antibody-independent mechanisms.

5.5 Immunomodulatory Drugs for the Treatment of the Nephrotic Syndrome

The efficacy of immunomodulatory drugs for treating various forms of the nephrotic syndrome provides important support for the immunologic basis of these diseases. The standard treatments for MCD and FSGS include corticosteroids, calcineurin inhibitors (CNIs), and mycophenolate mofetil. There is also evidence suggesting that cyclophosphamide, rituximab, and adrenocoricotropic hormone are effective in some patients [53]. For some of the drugs listed above, the reduction in proteinuria may be independent of the effects on the immune system. For example, CNIs and rituximab may act directly on the podocytes [54, 55]. It is striking, however, that multiple different immunomodulatory drugs with different mechanisms of action are effective in subsets of patients with MCD and FSGS. None of the drugs is effective in all patients, though, likely reflecting the underlying heterogeneity of the diseases. Regardless, until the pathophysiology of glomerular injury in these diseases is better understood, the therapeutic mechanisms of these different drugs will remain uncertain.

For MN, the mechanisms by which immunosuppressive medications ameliorate disease are more straightforward. Drugs that prevent or reduce the production of nephritogenic antibodies will block the underlying process that causes the disease. Most regimens include corticosteroids, and the response to these agents is often rapid. Improvement in MN can take months, however, likely because immunosuppressive drugs do not affect pre-existing nephritogenic antibodies in the circulation or ICs already deposited in the glomerular capillary wall. Thus, there is rationale for inclusion of agents that rapidly block the pro-inflammatory mediators of injury (e.g. complement activation and FcR signaling) and that prevent the formation of nephritogenic antibodies (e.g. B cell and plasma cell-targeting therapies) in the treatment of IC-mediated glomerular diseases.

5.6 Conclusions

Proteinuria is caused by the disruption of the glomerular filtration barrier, and several features of glomerular architecture make it particularly susceptible to inflammatory injury (Fig. 5.2). Resident glomerular cells express inflammatory cytokines, immune receptors, adhesion molecules, and co-stimulatory molecules. Therefore, there is likely continuous interaction between the immune system and the glomerulus. Indeed, a large number of systemic events that engage the immune system, such as infections or autoimmunity, are associated with the transient development of proteinuria or the full nephrotic syndrome. Although some of these immune-mediated mechanisms of injury are consequences of the unique architecture of the glomerulus, other connections between systemic immunity and proteinuria seem to be caused by an active response of glomerular cells to immune signals, such as TLR ligands, and may be adaptive in some settings. There is not yet a teleological

explanation for why the glomerulus expresses the machinery to sense and to respond to these immunologic signals, but one intriguing possibility is that the kidney is an "immunologic organ" and participates in the response to pathogens.

Experimental tools and animal models have been useful for studying the role of the immune system in the pathogenesis of proteinuria. Studies in which human products are injected into rodents have provided compelling evidence of soluble factors that increase proteinuria. The Heymann nephritis model of MN has provided important insights into the role of ICs in the development of podocyte injury, and many of the molecular and histologic features of the model are nearly identical to human MN. Studies in humans have revealed specific target antigens and autoantibodies that are both biomarkers of and likely contributors to the pathogenesis of the diseases that cause the nephrotic syndrome. Thus, future clinical trials can employ these markers to monitor the response of patients to immunomodulatory treatments. Furthermore, the identification of specific target antigens may lead to the development of therapies that directly block the immunologic causes of disease.

Monoclonal antibodies have been developed to block specific cytokines and to deplete T cells or B cells, and a growing number of recombinant proteins have entered clinical use. The effects of these different drugs—some salutary, some malign—have improved our understanding of the pathogenesis of proteinuria. New strategies for blocking or amplifying particular components of the immune system hold great therapeutic promise for these diseases and will undoubtedly improve the treatment of the nephrotic syndrome.

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Chapter 6
Minimal Change Disease

Gabriel M. Cara-Fuentes, Richard J. Johnson, and Eduardo H. Garin

Abbreviations

ACTH	Adrenocorticotrophic hormone
Angptl-4	Angiopoietin-like 4
APN	Arbeitsgemeinschaft für Pädiatrische Nephrologie
ASMase	Acid-sphingomyelinase
BUN	Blood urea nitrogen
CCT	Cortical collecting tubule
CD80	Cluster of differentiation 80
CNI	Calcineurin inhibitor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4.
ENaC	Epithelial sodium channel
FDA	Food and Drug Administration
FP	Foot processes
FR	Frequent relapsing
FR	Frequent relapsing
FSGS	Focal segmental glomerulosclerosis
g	Grams
GBM	Glomerular basement mkembrane
HSP	Heparan-sulfate proteoglycans
IFN	Interferon
Ig	Immunoglobulin.

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IL	Interleukin
INS	Idiopathic nephrotic syndrome
ISKDC	International Study of Kidney Disease of Children
IV	Intravenous
kDa	Kilodalton
KDIGO	Kidney Disease: Improving Global Outcomes
kg	Kilograms
LPS	Polysaccharide
ManNAc	Acetylated N-acetylmannosamine
MC1R	Mineralcorticoid-1 receptor
MCD	Minimal Change Disease
mEq	Milliequivalents
mg	Milligrams
MMF	Mycophenolate mofetil
PA	Puromycin aminoglycoside
PBMC	Peripheral mononuclear cells
PDGF	Platelet-derived growth factor
PHN	Passive Heymann nephritis
pI	Isoelectric point
Poly:IC	Polyinosinic:polycytidylic acid
RCT	Randomized controlled trial
SCID	Severe combined immunodeficient
SD	Steroid-dependent
SMPDL-3b	Sphingomyelin phosphodiesterase acid-like 3 b protein
TG	Transgenic
TLR	Toll-like receptor
TNF	Tumor necrosis factor
URI	Upper respiratory infection.
VEGF	Vascular endothelial growth factor
Vs	Versus

6.1 Introduction

Minimal Change Disease (MCD) refers to a type of nephrotic syndrome characterized by the presence of podocyte foot process fusion on electron microscopy and by the absence of major structural glomerular changes and immune deposits on light microscopy and immunofluorescence respectively [1]. The terms lipoid nephrosis, minimal lesion, nil disease, have been used as synonyms for MCD.

Among the different types of idiopathic nephrotic syndrome, MCD displays a higher rate of remission to steroids and the best long-term outcome despite the relapsing course of the disease observed in most of MCD patients [2].

6.2 Epidemiology

MCD is the most common type of nephrotic syndrome in children and accounts for about 97 % of all cases with nephrotic syndrome under 4 years of age. After that, the frequency decreases steadily reaching about 50 % of all cases between 8 and 16 years of age [3]. The incidence of MCD in <16 years has been estimated at 2–7 cases/100,000 and the prevalence at 16/100,000. MCD is more predominant in boys (2:1) during childhood but presents with a similar gender distribution among adolescents [4]. In adults, MCD represents only the 20 % of cases of nephrotic syndrome [5].

6.3 Etiology

In the majority of patients, especially in children, MCD is a primary glomerular disease. Secondary causes are shown in Table 6.1 [6]. Relapses in patients with MCD have been reported to follow exposure to inhaled allergens, foods, insect stings, and vaccinations. It is clear that some patients with MCD may present with nephrotic syndrome after an allergen exposure, and many patients with MCD have increased serum Immunoglobulin (Ig) E levels. Although allergens occasionally have been implicated in triggering nephrotic syndrome in patients with MCD, evidence that blocking the specific allergic agent may prevent relapse is weak. This suggests that the atopic response is associated with the immune abnormality in patients with MCD, rather than having a causal role [6].

A strong clinical association has been established between MCD and Hodgkin's disease. MCD represents the most common form of nephrotic syndrome in

Table 6.1 Secondary causes of Minimal Change Disease

Drugs
Antimicrobials: ampicillin, cefixime, rifampicin
NSAIDS: ibuprofen, naproxen, zomepirac, indomethacin, fenoprofen, piroxicam, diclofenac
Lithium
Probenecid
Penicillamine
Neoplasms
Hodgkin's lymphoma
Non-Hodgkin's lymphoma
Thymoma
Infections
Syphilis
Tuberculosis
Mycoplasma
Atopy
Not proven cause-effect: Pollen, dairy product, bee sting, poison oak/ivy

Hodgkin's disease [7]. However, the incidence of MCD remains very low in patients with Hodgkin's disease (0.4 %) [8, 9]. Overexpression of C-mip, an 86-kDa protein recruited to lipid rafts, has been found in Reed-Sternberg cells and podocytes from those patients with Hodgkin's disease who developed MCD [10]. C-mip has been suggested to interfere with nephrin phosphorylation by blocking the tyrosine kinase Fyn interaction with nephrin, leading to cytoskeleton rearrangement and proteinuria. Although the observation is tantalizing, it remains to be determined whether C-mip displays a casual role in MCD in Hodgkin's disease or may represent an epiphenomenon in the context of podocyte injury.

A study on polymorphisms of interleukin-4 (IL-4) related genes found a lower frequency of the T allele in MCD patients than controls. IL-4 has been associated with atopy. However, the above study did not include any patient with atopy in the control group whereas 7 out of 57 MCD patients presented with asthma or atopy [11]. Thus, the clinical significance of IL-4 polymorphism is unclear. The prevalence of a CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) polymorphism (+49GG genotype), associated with decreased expression of CTLA-4, is significantly increased in MCD patients compared to normal controls [12]. Impaired CTLA-4 production may play a role in proteinuria as CTLA-4 is the natural inhibitor of CD80, a key molecule in the development of proteinuria in MCD.

MCD is rarely seen in more than one member of a single family. In such cases, MCD may present with a pattern of steroid dependence or resistant, which altogether may suggest an underlying mutation in any of the gene encoding for slit diaphragm proteins.

6.4 Pathogenesis

MCD has been assumed to be result of a dysregulation of the immune system. Shalhoub, in 1974, postulated MCD to be a T-cell disorder by which a circulating cytokine(s) released by T cells leads to increase permeability of glomerular basement membrane (GBM) to plasma protein and proteinuria [13]. This hypothesis was founded on some clinical and pathological observations: (1) absence of electron-dense deposits, immunoglobulins and complements in MCD patients' glomeruli, (2) remission of proteinuria induced by steroids and cyclophosphamide or spontaneous remission induced by measles; thought to be mediated by suppression of cell mediated immunity, (3) association of MCD with Hodgkin's disease. This hypothesis led to investigators to search for the pathogenic circulating cytokine by using different approaches (see below).

6.4.1 Circulating Cytokines in MCD

Studies of cytokines in serum from INS patients have often showed variable results. Thus, serum levels of IL-2, IL-4 and interferon (IFN) -γ have been reported to be high, low or unchanged in patients with idiopathic nephrotic syndrome (INS) during relapse compared to those in remission [14–16]. While some authors have reported

increased serum levels of IL-2 receptor, IL-5, IL-10, TNF (tumor necrosis factor) -α, VEGF (vascular endothelial growth factor) in INS patients during relapse, others did not find differences in these interleukins among those patients in relapse compared to those in remission [17–20]. The discrepancy among studies may be explained by the lack of kidney biopsy in many patients, therefore including patients without MCD, lack of standardization of assays among authors or the concomitant use of immunosuppressive therapy. An alternative explanation is that the above mentioned cytokines do not play a role in MCD.

Of all cytokines, IL-8 and IL-13 have been suggested to play a major role in proteinuria in MCD. We [21] and others [22] have shown that IL-8 is increased in serum in MCD patients during relapse when levels are compared to those patients in remission. These patients also present with increased IL-8 mRNA on unstimulated peripheral blood mononuclear cells (PBMC) during relapse. In addition, IL-8 infusion into rats resulted in increased ^{35}sulfate (isotope) uptake by the glomerular basement membrane (GBM), decreased in glycosaminoglycans and albuminuria. Finally, the addition of anti-IL8 antibody abolished the changes associated with IL-8 infusion in the rat. These findings suggest a causal link between circulating IL-8 and proteinuria caused by an augmented glycosaminoglycans catabolism [23]. In contrast, infusion of other cytokines, such as VEGF, TNF-α or platelet-derived growth factor (PDGF), into rats did not result in proteinuria. Overall, there is strong clinical and experimental evidence supporting a role of IL-8 in proteinuria in MCD through its effect on GBM glycosaminoglycans.

A role of circulating IL-13 in the pathogenesis of proteinuria has been suggestive by studies in an IL-13 transgenic (TG) rat model reported by Lai [24]. These TG rats developed increased levels of serum IL-13 followed by glomerular expression of CD80 (a costimulatory surface protein), podocyte effacement and proteinuria. Of interest, in this animal model, serum IL-13 did not correlate with proteinuria. The clinical significance of the TG model of IL-13 is challenged by the lack of consistent serum IL-13 levels in MCD. Some investigators have reported increased IL-13 levels in serum of INS patients [16, 18]. However, in some of these studies, serum levels of IL-13 were higher in INS patients during remission than during relapse, questioning the presumed pathogenic role of circulating IL-13. It is important to emphasize the often concurrence of atopy or asthma in patients with INS. The increased IL-13 serum levels could be related to the underlying immune process (atopy or allergy) or upper respiratory infection rather than being the cause of proteinuria in MCD. Furthermore, we [25] and others [26] found no detectable IL13 levels in serum or unstimulated PBMC from MCD patients during relapse.

6.4.2 Mechanism(s) of Proteinuria

6.4.2.1 Role of Capillary Wall Anionic Charges

MCD is characterized by an increased glomerular permeability to plasma proteins, mostly albumin. This has been historically attributed to a defective glomerular charge-selective barrier caused by the loss of fixed negative charges in the capillary wall [27]. This was based on the following observations: (1) the permeability,

estimated by fractional clearance studies, of anionic dextran sulfate was lower than those dextran molecules with neutral charge despite sharing a similar size and conformation. In contrast, permeability was enhanced by using cationic dextran molecules [28], (2) Diminished uptake of colloidal iron, a polycation, by glomeruli from MCD patients in comparison to controls, suggesting loss of glomerular polyanion [29].

6.4.2.2 Glomerular Capillary Wall and Anionic Charges

The glomerular capillary wall is composed of three layers (see Fig. 6.1):

(a) **Endothelium:** Endothelial cells form a thin, fenestrated layer that lines the inner portion of the capillary lumen. This layer is covered by polyanionic proteins that provide negative charge to its surface. The endothelium does not

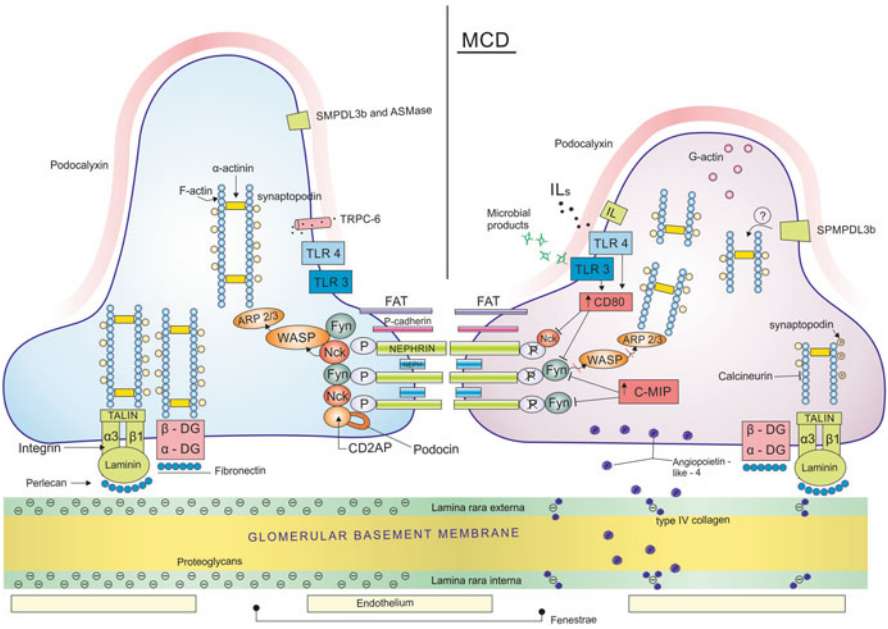

Fig. 6.1 Molecular anatomy of the podocyte foot process (FP) and actin cytoskeleton in healthy state (*left*) and in Minimal Change Disease (MCD) during relapse (*right*). In MCD, microbial products and/or interleukins (ILs) target toll-like receptors (TLR) promoting CD80 and C-mip expression which in turn reduce nephrin phosphorylation by binding Nck and/or Fyn proteins. This may result in a dysregulation of the downstream pathway that links nephrin with cytoskeleton resulting in rearrangement of the actin cytoskeleton. SMPDL-3b, sphingomyelin phosphodiesterase acid-like 3 b protein; ASMase, acid-sphingomyelinase; ARP 2/3, actin related protein 2/3 complex; WASP, Wiskott-Aldrich syndrome protein; CD2AP, CD2-Associated Protein; P, phosphorylated site; DG, dsytroglycans

appear to play a key role in MCD since its large fenestrations (70–100 nm diameters) may allow the passage of large proteins in normal circumstances [30].

(b) **Glomerular basement membrane**: It is composed of a central dense layer or lamina densa which is surrounded by 2 electro-lucent layers named lamina rara interna and externa when in contact with endothelial or epithelial cells respectively [31]. The major components of the GBM are type IV collagen and heparin-sulfate proteoglycans (HSP) including laminin, perlecan and agrin. HSP provides anionic charges to the GBM, which has been considered the defective charge-selective barrier in MCD for years. Indeed, a decreased number of anion sites and heparin sulfate molecules have been found in the GBM of MCD patients [32]. Angiopoietin-like 4 (Angptl-4, see below) and IL-8 have been suggested as mediator of negative charges in the GBM. As previously mentioned, IL-8 augments proteoglycans catabolism in GBM leading to a reduction of anionic charges and mild proteinuria.

However, the causative link between the reduction of anionic sites in GBM and development of proteinuria has been challenged by recent studies. Two knock-out mice models, characterized by the absence of agrin and perlecan and the reduction of heparin sulfate respectively, showed no proteinuria or mild proteinuria despite a significant reduction in GBM's anionic charges [33]. In a different transgenic model, the overexpression of heparanase resulted in a reduction of anionic sites in GBM but mice did not develop proteinuria [34].

(c) **Podocytes:** Podocytes are highly differentiated visceral epithelial cells that are anchored to GBM by integrins and are covered by a surface coat rich in sialoglycoproteins (podocalyxin) on its apical side and slit diaphragm. The role of integrins and podocalyxin in MCD remain uncertain. Thus, podocytes remain attached to the GBM in nephrotic states questioning a role of integrins in MCD. Expression of podocalyxin has been found reduced in MCD by some authors [35], but not by others [36], arguing against a presumed causative role of podocalyxin in MCD.

6.4.2.3 MCD as a Podocyte Disease

Recent advances in the molecular composition of podocytes and slit diaphragm have suggested a shift in the pathogenesis of proteinuria in MCD from the concept of MCD as immune systemic disease to MCD as a podocyte disease [37].

The current hypothesis is that, in MCD, podocyte reacts to different stimuli such as viral particles, allergens, and/or cytokines leading to structural changes at the level of the slit diaphragm causing proteinuria [38].

Podocytes can be divided into three structural and functional segments: cell body, major processes and foot processes (FP). Neighboring podocyte foot processes interdigitate forming filtration slits of ~30–40 nm width, that are bridged by a thin zipper-like pattern membrane denominated slit diaphragm. Nephrin, the most abundant protein of the slit diaphragm, plays a key role in formation and maintenance of slit diaphragm by providing structural support and by regulating signaling pathways linking the slit diaphragm to the actin cytoskeleton.

There is recent evidence supporting CD80, a transmembrane protein expressed by human podocytes as the key molecule leading to foot processes effacement and proteinuria. CD80 overexpression by podocytes and proteinuria was observed in LPS (lipopolysaccharide)-injected mice. However, no proteinuria was observed in CD80 knock-out mice after LPS injection. These observations were independent of T cells as SCID (severe combined immunodeficiency) mice exposed to LPS also developed foot processes effacement and proteinuria [39]. The same pattern of increased CD80 podocyte expression and proteinuria have been observed in the Poly:IC (Polyinosinic:polycytidylic acid) and IL-13 animal models [24, 40].

LPS and Poly:IC stimulate podocyte CD80 expression by activation of podocyte toll-like receptors (TLR) 4 and 3 respectively [41]. This is of great relevance as relapse in MCD patients is often triggered by viral respiratory infections and circulating viral particles are known to stimulate TLR-3. Up to 10% of healthy children developed proteinuria during febrile illnesses. In this cohort of patients, we have found increased urinary CD80 excretion compared to afebrile healthy children (unpublished observations). These findings would support the role of circulating microbial products, rather than interleukins, as the trigger of CD80 podocyte expression and proteinuria in MCD patients. In addition, we have found increased podocyte CD80 expression by cultured podocytes after stimulation with sera from MCD patients in relapse when compared to those in remission [25]. No such increased CD80 podocyte expression was observed when podocytes were stimulated with supernatants from cultured PBMC from MCD patients in relapse suggesting the presence of a circulating factor in the serum of these patients rather than a factor released by PBMC.

The above experimental findings suggesting a key role of podocyte CD80 as mediator of proteinuria in MCD are supported by growing clinical evidence. We [42–45] and others [46] have consistently found, in different cohorts of patients, an increased urinary CD80 excretion in MCD patients during relapse compared to those MCD in remission, control subjects, or FSGS patients with massive proteinuria. Urinary CD80 excretion normalized in MCD patients once remission is achieved. The source of the urinary CD80 in MCD patients is likely the podocyte based on the following facts: (1) although serum level of soluble CD80 is decreased in patients with MCD during relapse compared to MCD in remission suggesting increased urinary losses, urinary CD80 in MCD patients has a molecular weight of 53 kilodaltons (kDa), consistent with it being the cell-membrane associated CD80, in contrast to soluble CD80 (23 kDa) that is present in the circulation [42, 43]. (2) CD80 podocyte expression in MCD patients in relapse is increased when compared to expression in MCD patients in remission [43].

We have hypothesized that MCD patients in relapse present a persistent CD80 podocyte expression due to an impaired production of podocyte CTLA-4, the natural inhibitor of CD80, suggesting that MCD is the result of an autocrine podocyte dysregulation [45]. We have found that urinary CTLA-4 excretion is increased in MCD patients during relapse compared to those in remission. However, urinary CTLA-4 levels did not correlated with urinary CD80, suggesting a suboptimal CTLA-4 response to the increased podocyte CD80 expression observed in MCD. More important, a polymorphism in the CTLA-4 gene, associated to

reduced CTLA-4 production, has been reported to be more frequent in MCD patients than in control subjects [12]. Finally, administration of CTLA4-IgG1 to a MCD patient with high urinary CD80 excretion and undetectable urinary CTLA-4, resulted in plummeting of urinary CD80 at 24 h followed by resolution of proteinuria during 9 days [47]. These findings provide strong clinical evidence of the causative link between podocyte CD80 expression and proteinuria in MCD, as suggested by experimental studies.

The mechanism(s) by which CD80 induces cytoskeleton changes and proteinuria remains unresolved. We have found increased CD80 expression and decreased phosphorylated nephrin expression in podocytes exposed to sera from MCD in relapse (poster communication, ASN 2014). However, nephrin phosphorylation was preserved when human podocytes were pre-incubated with CTLA-4, prior to be cultured with sera from MCD patients. This finding suggests that podocyte CD80 may reduce nephrin phosphorylation, likely by disrupting binding of the tyrosine kinases Fyn and/or Nck binding to nephrin (Fig. 6.1). In addition, we and others have found reduced glomerular phosphorylated nephrin by immunohistochemistry in MCD patients [48].

Human Angptl-4, a 45–65 kDa glycoprotein has been proposed as a mediator of proteinuria in MCD by Chugh et al. [49]. They found glomerular expression of Angptl-4 increased in several animal models of proteinuria including the LPS, puromycin aminoglycoside (PA), Buffalo Mna rats and passive Heymann nephritis (PHN).

LPS-injected wild-type mice developed higher albuminuria than that observed in Angptl-4 knock-out mice (Reference). A transgenic rat model that overexpressed Angptl-4 in podocytes developed by these authors had marked loss of GBM heparan sulfate proteoglycans associated with foot processes effacement and albuminuria at 3 months of age compared to control rats. Proteinuria was reduced by treating tap water with acetylated N-acetylmannosamine (ManNAc), a sialic acid precursor, by shifting Angptl-4 from a high to a neutral isoelectric point (pI). Based on these observations, the authors proposed that in MCD, podocytes secrete Angptl-4 (mostly with pI > 8) that migrates to the GBM and endothelium resulting in reduced GBM anionic charges and, therefore, proteinuria [49]. In a subsequent study, the same group reported increased plasma levels of Angptl-4 in animal models of proteinuria and in patients with nephrotic syndrome secondary to MCD, focal segmental glomerulosclerosis (FSGS), non-HIV collapsing nephropathy and membranous nephropathy [50].

Data on Angptl-4 in MCD patients during relapse are scarce and only reported by the same group. Angptl-4 oligomers (220 kDa) with pI <8 were detected in urine in 4 MCD patients. One of these patients also had urinary Angptl-4 molecules (55–70 kDa) with a pI > 8. In addition, Angptl-4 oligomers (100–160 kDa) with pI < 8 and Angtpl-4 molecules (55–70 kDa) with pI > 8 were also detected in blood of few MCD patients [49]. In addition, Angptl-4 was mildly expressed, by immunofluorescence, in kidney tissue from 5 MCD patients in relapse compared to control. Angptl-4 colocalized with podocyte, GBM and endothelial cell markers.

In contrast we have found that urinary Angptl-4 excretion does not correlate with proteinuria in a large cohort of patients with MCD, FSGS and membranous

nephropathy. Higher urinary Angptl-4 levels were found in few MCD patients during relapse compared to control subjects (unpublished observations). In contrast to that suggested by Chugh, the source of Angptl-4 detected in urine in MCD is unlikely to be from the podocyte because: (1) we found no or minimal Angptl-4 staining by immunofluorescence in kidney tissue from our MCD patients in relapse, (2) no increased Angptl4 expression was observed in human podocytes cultured with sera from MCD patients in relapse compared to those exposed to sera from MCD patients in remission, (3) contrary to a previous study, Angptl-4 is lower in serum from MCD patients in relapse compared to those in remission. More importantly, Angptl-4 detected in urine from our MCD patients in relapse had a pI of 5.4, in contrast to the high pI reported by others, challenging the significance of the role of charges or pI in the development of proteinuria.

Overall, our findings from a large and well-defined cohort of MCD patients do not support a role of podocyte Angptl-4 in proteinuria in MCD. It seems that urinary Angptl-4 in these patients is the result of a defective glomerular filtration barrier to plasma proteins.

6.5 Pathological Features

MCD is a glomerular disease. On **light microscopy** the glomeruli show no or "minimal" abnormalities. Some specimens may depict slight increase in mesangial matrix and cellularity. Visceral epithelial cells look normal. The presence of hypertrophied or proliferation of podocytes as well as capsular adhesion are not consistent with MCD.

Globally sclerotic glomeruli in contrast with glomeruli showing segmental sclerosis are also consistent with a diagnosis of MCD only if they represent a small number (<5 %) of total glomeruli and are not associated with surrounding tubular atrophy and/or interstitial fibrosis [51].

Although natural senescence of glomeruli may be observed, their clinicopathological interpretation is difficult since there are no well-defined standards for the number of glomeruli affected in normal subjects with age, especially for those under the age of 20 years [52].

Tubules and interstitium show no specific lesions. However, a few cases may present mild focal tubular atrophy, mild segmental interstitial fibrosis, or inflammation. Fat and hyaline droplets may be found in the proximal tubule.

By **immunofluorescence microscopy**, the glomeruli are usually negative for immunoglobulins and complement components. If present they are low in intensity, confined to the mesangium, and not associated with electron dense deposits. A few specimens may have diffuse mesangial Ig M staining which have led to some authors to define it as a different glomerular disease (IgM nephropathy). However, the current consensus regards the presence of IgM in these patients' glomeruli as inconsequential [3]. Similarly, predominant C1q deposits have been occasionally described in mesangium from patients with idiopathic nephrotic syndrome. The significance of

these C1q deposits, in the absence of immune complex disease such as systemic lupus erythematosus is not clear. The prognosis appears to be associated to the lesions observed on light microscopy, MCD or FSGS, rather than to the intensity of C1q staining.

On **electron microscopy**, the only consistent glomerular finding in MCD is the effacement of the foot processes. The degree of effacement is related to the activity of the disease. The effacement of the foot processes is not pathognomonic of MCD and can be seen in other conditions presenting with massive proteinuria. The podocytes are firmly attached to the GBM while the slit pore density is decreased resulting in a decreased in the filtration surface area between podocytes [53]. The GBM is usually normal and no electron dense deposits are observed.

6.6 Clinical Manifestations

The main clinical manifestation of MCD is edema. In MCD, edema is pitting, of sudden onset and gravitational (periorbital in the morning and more evident on legs/ankles in the evening). MCD patients may present with massive anasarca that may lead to pleural and/or pericardial effusion resulting in respiratory distress and bowel edema causing diarrhea. There is a strong correlation between the severity of the edema and serum levels of albumin.

MCD patients may develop abdominal pain and emesis as the result of bowel ischemia due to severe hypovolemia. In addition, hypovolemia may lead to orthostatic hypotension or renin-mediated hypertension. These symptoms are alleviated by expanding the intravascular compartment by intravenous albumin infusion. The presence of severe anasarca and/or hypovolemia is a risk factor for acute pancreatitis, renal thrombosis and spontaneous bacterial peritonitis. The presence of macroscopic hematuria argues against MCD as underlying glomerular disease or may be the result of renal vein thrombosis.

The mechanism(s) of edema formation in MCD remains to be defined. It has been proposed that the massive proteinuria leads to hypoalbuminemia and decreased intravascular oncotic pressure [54]. As a result, fluid shifts from the intravascular to the interstitial compartment leading to edema and intravascular depletion. The hypovolemia results in renal hypoperfusion which activates the renin-angiotensin-aldosterone axis and sympathetic nervous system. Indeed in MCD patients there is evidence of hypovolemia as well as increased renin and aldosterone serum levels. The elevated circulating levels of renin, aldosterone and norepinephrine in turn increase the proximal and distal tubular reabsorption of sodium.

There are several arguments that argues against this hypothesis: (1) analbuminemic rats do not develop edema or sodium retention [55], (2) natriuresis and loss of edema that take place during remission occur when patients are still hypoalbuminemic [56], (3) no diuresis is observed after expansion of intravascular compartment with albumin unless a diuretic is given, (4) bilateral adrenalectomy or antialdosterone agents do not reverse sodium retention in MCD patients [57].

The current evidence suggests increased sodium reabsorption in the distal tubule as the primary mechanism leading to edema. Increased reabsorption is triggered by massive proteinuria. This hypothesis is supported by findings in puromycin aminoglycoside rat model of nephrotic syndrome. Ichikawa et al. [58] selectively infused puromycin in one kidney and measured urinary sodium concentration in renal tubules from both rat kidneys by micropuncture technique. A similar amount of sodium was found at the end of distal convoluted tubule in both rat kidneys. However, the final sodium concentration in urine was three times lower in the kidney exposed to PA compared to control kidney, suggesting sodium reabsorption in the cortical collecting tubule (CCT). The latter has been attributed to an increased number of open epithelial sodium channels (ENaC) in the CCT, likely due to the removal of the ENaC gamma inhibitory domain by urinary plasmin [59]. Other mechanisms for sodium retention may be also operating since if edema is solely due to ENaC activation, one might have expected a substantial diuretic response with amiloride, a competitive inhibitor of ENaC. However, no such diuretic response to amiloride is observed in MCD during relapse. In addition, sustained activation of ENaC as observed in Liddle syndrome is associated to sodium retention but no edema formation.

6.7 Laboratory Tests

6.7.1 Urinalysis

Nephrotic range proteinuria has been defined in different ways. Thus, proteinuria greater than 50 mg/kg in a 24 h urine collection in children or greater than 3.5 g/24 h in adolescents and adults, is considered nephrotic range proteinuria. In children, the International Study of Kidney Disease of Children (ISKDC) defined nephrotic range proteinuria as proteinuria greater than 40 mg/h/m^2 or 200 mg protein/mmol urine creatinine. ISKDC definition is based on urine collections obtained overnight and not in a 24 h period. Proteinuria in MCD is highly selective, with a predominance of albumin compared to immunoglobulins or lower molecular weight proteins.

Hyaline casts and fat bodies are often observed in the urine sediment of MCD patients. These bodies are formed by precipitation of albumin and lipoproteins, respectively, with Tamm-Horsfall mucoprotein secreted by renal tubule cells.

Microscopic hematuria may be observed in 20–30 % of patients, most of who present with signs of hypovolemia. Thus, it has been thought that hematuria may be consequence of renal ischemia as hematuria resolves once remission is achieved.

6.7.2 Hypoalbuminemia

Hypoalbuminemia is the hallmark of nephrotic syndrome. The main mechanism leading to low serum albumin levels is the increased glomerular filtration permeability to plasma proteins. In addition, it is controversial if there is also an

increased catabolism of filtered albumin by proximal tubule cells [60]. The current evidence suggests that albumin is reabsorbed intact at that level. The liver production of albumin is increased in MCD patients and gastrointestinal losses are minimal.

6.7.3 Hyperlipidemia

Hyperlipidemia is a common feature of patients with nephrotic syndrome. It is the result of an increase hepatic synthesis of cholesterol and triglycerides and a decreased catabolism of lipoproteins. The latter is due to increased urinary losses of albumin which leads to an excess of fatty acids in plasma that will inhibit lipoprotein lipase in fatty tissues and serum. In addition, urinary losses of lecithin cholesterol acyltransferase lead to a reduction of chylomicrons and VDLD clearance.

6.7.4 Hematology

Elevated hematocrit is often observed in MCD patients during relapse as many of them present with signs of hypovolemia. Those patients with persistent nephrotic syndrome may develop normocytic-normochromic anemia. This is not due as initially thought to transferring iron losses resulting in iron deficiency because iron stores are normal in most of MCD patients and anemia is not hypochromic. The anemia in MCD has been shown to be due to erythropoietin deficiency (Reference).

6.7.5 Electrolytes

Serum electrolytes are usually within the normal range. Hyponatremia is occasionally observed in MCD. It may represent dilutional hyponatremia or pseudohyponatremia, which is found in those patients presenting with severe hypercholesterolemia. In these cases, a normal serum osmolality will make the diagnosis of pseudohyponatremia. Diuretic therapy may also result in hyponatremia and together with fluid restriction may lead to hypernatremia due to free water deficit.

6.7.6 Calcium and Vitamin D

Total calcium is consistently decreased in serum of MCD patients during relapse as result of hypoproteinemia. However, ionized calcium is usually within normal range. Urinary losses of vitamin D metabolites binding proteins result in lower serum concentration of these metabolites. However, symptomatic hypocalcaemia or

bone disease (except for that associated to the prolonged use of steroids) is rarely seen in MCD patients. Thus, there is no indication to use calcium or vitamin D supplement routinely in MCD patients.

6.7.7 Complement and IgG Levels

Some patients with MCD lose immunoglobulin in their urine and may have hypogammaglobulinemia. A loss of certain complement proteins such as factor B and properdin may also occur [61]. As a consequence, subjects with MCD are at increased risk for bacterial infections, especially from encapsulated organisms. Interestingly, serum C3 levels are elevated in MCD [61].

6.7.8 Serum Creatinine and Blood Urea Nitrogen

Serum creatinine and blood urea nitrogen (BUN) are usually within normal range in MCD patients. However, mild increased in both serum markers may be found in those MCD patients who present with marked hypovolemia during relapse. In those cases, restoration of normovolemia after albumin infusion will result in normalization of serum creatinine and BUN.

6.8 Natural Course of the Disease

The natural course of MCD before steroid therapy was available cannot be defined because the absence of histological diagnosis in all cases with nephrotic syndrome. There have been only two groups that have compared the long-term outcome of biopsy-proven MCD patients treated with and without prednisone. The study by Black et al. [62] in adults included a total of 31 patients. The remission rate at 4-year follow-up was similar among patients treated with and without steroids. About 50 % of patients in the control group underwent spontaneous remission at 15 months follow-up with 20 % having persistent proteinuria after 4 years.

Coggins [63] compared the long-term outcome of adults with MCD treated with an alternate regimen of prednisone (average 125 milligrams (mg)/day for 2 months) compared to a placebo group. In agreement to the study published by Black, the percentage of patients achieving complete remission was not statistically different among the 2 groups (93 % vs 64 %, prednisone vs placebo respectively) at 77 months follow-up.

Similarly, Coggins observed that 6 of 14 MCD patients receiving prednisone (125 mg on alternate days for 2 months) underwent complete or partial remission at 3 months compared to none of 14 patients receiving placebo. However, the long-term outcome of patients from these 2 studies is quite interesting. Of those patients not receiving prednisone, 50 % underwent spontaneous remission at 15 months [62]

and 80 % and 64 % of patients had proteinuria <1 gram (g)/day at 48 and 55 months respectively [62, 63]. In addition, the rate of remission at 4-year follow-up was similar among patients treated with and without steroids.

Spontaneous remission has been described in children with idiopathic nephrotic syndrome (no histological diagnosis) at the onset of the disease [64] (2 %) or in subsequent relapses [65] (31 %). Wingen et al. [66] studied the course of 15 and 17 patients with frequently relapsing and steroid dependent idiopathic nephrotic syndrome (see definitions in Table 6.2) [2] for a mean period of 6 years. Most of patients underwent at least 1 spontaneous remission. Moreover, 23 % and 10 % of relapses spontaneously resolved in frequent relapsing and steroid dependent idiopathic nephrotic syndrome patients respectively within 2 weeks. These findings led to authors to recommend delaying steroid therapy for few days in the absence of progressive clinical signs to minimize steroid exposure.

6.8.1 MCD and Upper Respiratory Tract Infections

Upper respiratory infections (URI) seem to trigger relapses in up to 80 % cases [67]. It is currently thought that circulating viral particles may stimulate toll-like receptors (TLRs) on podocytes resulting in podocyte CD80 overexpression which in turn leads to cytoskeleton rearrangement, opening of slit diaphragm and proteinuria [38, 39]. Interestingly, viremia may be also present in subclinical upper respiratory infections and asymptomatic patients.

A leaky glomerular filtration barrier has been proposed as an innate defense mechanism in order to accelerate the shedding of circulating viral particles. Not surprisingly, febrile illnesses are not only associated with a full relapse in MCD but also can result in transient proteinuria in otherwise healthy patients. Therefore, in an MCD patient, it is critical to distinguish between transient proteinuria as result of URI from a full relapse in order to minimize steroids exposure.

Table 6.2 Clinical definitions in idiopathic nephrotic syndrome

Remission: Three consecutive days of trace or negative proteinuria on dipstick or rate of urinary excretion of protein <4 mg/h/m^2
Initial responder: Attainment of complete remission within initial 8-week of corticosteroid therapy (4 week for KDIGO 2012)
Relapse: Three consecutive days of ≥3+ proteinuria on dipstick or reappearance of proteinuria ≥40 mg/h/m^2
Steroid resistance: failure to achieve remission after an 8-week course of corticoid therapy
Infrequent relapse: One relapse within 6 months of initial response, or one to three relapses in any 12-month period
Frequently relapse: Two or more relapses within 6 months of initial response, or four or more relapses in any 12-month period
Steroid dependence: Two consecutive relapses during corticoid therapy, or within 14 days of ceasing therapy

MCD patients may develop proteinuria during or shortly after an URI. The rate of relapse during URI was reduced from 48 % to 18 % when patients received a 7-day course of daily prednisolone at the onset of upper respiratory symptoms [68].

6.9 Treatment

6.9.1 Symptomatic Therapy

6.9.1.1 Diet

Protein intake requirements in MCD patients are 100 % of the recommended daily allowance according to age-predicted stature. High-protein diets result in worsening proteinuria and hypoalbuminemia. In contrast, low-protein diet reduces proteinuria and hypoalbuminemia but leads to malnutrition. Fluids restriction is not advised during the acute phase of edema formation since fluids will aggravate the hypovolemia. If intravenous albumin infusion is administered during this stage, fluids need to be restricted to at least 2/3 of daily maintenance. The same guidelines for fluid restriction are recommended in those patients who are hemodynamically stable. A critical step to control edema formation and avoid steroids side effects is sodium restriction, with a maximum sodium intake of 2 milliequivalents (mEq)/kilograms (kg)/day in children and a 2 g/day sodium chloride diet in adolescents and adults. The sodium restriction is continued until steroids are tapered off.

6.9.1.2 Physical Activity

Reduced physical activity and bed rest may be recommended in those patients who develop severe anasarca or orthostatic hypotension.

6.9.1.3 Edema

Sodium restriction is the first action needed in order to reduce edema. Patients with severe anasarca may benefit of intravenous infusion of albumin 25 % (1 g/kg of dry weight) followed by diuretics. Albumin infusion will increase the intravascular oncotic pressure shifting fluid from the interstitium to the vascular compartment. Thus, a rise in blood pressure is often observed during albumin infusion enhancing renal perfusion. Interestingly, no massive diuresis is observed despite of fluid shifting unless diuretic therapy is used. Albumin is typically infused over a period of 2–6 h according to the patient's hemodynamic status—2 h if the patient is hypotensive, 4 h if normotensive and 6 h if initially hypertensive. Close monitoring of vital signs and clinical status is critical to determine the rate of albumin infusion. Blood pressure should be measured at 30 min to hourly intervals because albumin infusion may lead to severe hypertension and/or respiratory and cardiac failure.

There are different approaches on when to administer diuretic therapy (usually a loop or distal tubule diuretic) during and after albumin infusion. It is our policy to administer one dose of intravenous (IV) furosemide 1 mg/kg immediately after albumin infusion is completed and then every 8 h for the following 24 h as the oncotic effect of infused albumin lasts approximately 24 h.

6.9.1.4 Hyperlipidemia

Chronic hyperlipidemia is a risk factor for cardiovascular disease. However, hyperlipidemia resolves upon resolution of proteinuria, which is usually achieved in MCD patients. Therefore no statin therapy is routinely recommended in MCD patients.

6.9.1.5 Infections

Infections represent one of the most worrisome complications in nephrotic syndrome and they were the main cause of death prior to antibiotic therapy. The increased urinary losses of IgG and complement factors lead to an opsonization defect which puts nephrotic syndrome patients at high risk for Streptococcal pneumonia infections. Prophylaxis with oral penicillin may be indicated during relapse despite the increasing pneumococcal resistance to this antibiotic.

6.9.1.6 Immunizations

The use of immunosuppressive therapy enhances the risk for viral infection such as varicella. Thus, varicella and pneumococcal vaccination are critical in MCD patients. It is advised to administer these immunizations after 3 months off immunosuppression therapy in order to maximize the immunological response. The efficacy of the pneumococcal vaccine is based on the bacterial opsonization by anti-pneumococcal immunoglobulin. Low levels of this immunoglobulin are found during relapse of nephrotic syndrome, which may explain why pneumococcal peritonitis still occurs in immunized patients during relapse.

6.9.2 Treatment of MCD

6.9.2.1 Control of Proteinuria

The use of steroids in MCD was initially empirical and resulted in increased diuresis in nephrotic patients. Subsequently, their use was justified on the concept that MCD was a T cell disease in which T cells released a cytokine(s) leading to increased glomerular permeability to proteins (Shalhoub's hypothesis) [13].

However, the validity of this hypothesis remains uncertain 40 years later given the lack of evidence supporting a role of circulating cytokines in the pathogenesis of proteinuria in MCD.

Molecular discoveries made in the last 20 years have provided a better understanding of the glomerular filtration barrier and the role of podocytes in proteinuria [69]. Thus, the concept of MCD has shifted from what was initially thought to be a systemic disease to the current concept of podocyte disease [37]. One of the strongest arguments supporting a role of cytokines in MCD was the fact that steroids and cyclosporine induce remission of proteinuria in most patients. However, it has been shown that both steroids and cyclosporine exert a direct effect on podocytes through different mechanisms [48, 70]. A decreased in nephrin phosphorylation has been found in kidney tissue from MCD patients in relapse and from rats treated with puromycin aminoglycoside [48]. It has been suggested that dysregulation of nephrin phosphorylation, likely caused by podocyte CD80 (unpublished observations) or C-mip overexpression [10], leads to a sequence of events resulting in podocyte foot processes effacement. Interestingly, steroids enhance nephrin phosphorylation in cultured human podocytes [71]. On the other hand, cyclosporine blocks the calcineurin-mediated de-phosphorylation of synaptopodin, which is critical to maintain integrity of the actin cytoskeleton [70]. Therefore, both steroids and cyclosporine, aside from their known immunomodulating properties, may act at the level of the podocyte preserving its molecular structure.

Corticosteroids are the cornerstone therapy to induce remission of proteinuria in patients with MCD, leading to remission in up to 95% and 80–90% of children and adults respectively. Although steroid-sparing agents are often used in selected MCD patients, data on their use as first line therapies are scarce. An 8-week course with cyclophosphamide result in resolution of proteinuria in more than 70% of patients. Cyclosporine given to MCD patients induced remission in all patients in a period of 7–23 days [72]. In addition, the use of cyclophosphamide is limited given its well-known dose-related gonadal toxicity. Likewise, prolonged courses of cyclosporine may result in irreversible renal damage. Altogether, the use of steroid-sparing agents as first line therapy in MCD should be reserved for selective cases such as those diabetes mellitus.

Steroid therapy in MCD includes 2 different stages: (1) Induction phase, in which steroids are administered on a daily basis to induce remission, followed by (2) tapering phase, in which steroids are switched from daily to alternate regimen and tapered down.

Induction Therapy

In 1981, the ISKDC published the outcome of 471 children with nephrotic syndrome (363 patients with MCD) treated with steroids [2]. The ISKDC arbitrarily defined a treatment protocol for the induction phase of prednisone 60 mg/day/m^2—roughly equivalent to 2 mg/kg—(maximum of 80 mg/day) given in 3 divided doses for 4 weeks. In this study, most of the MCD patients (93.1%) underwent remission after an 8-week prednisone course. In fact, 94% of the patients labeled as responders underwent remission by the fourth week of with an average time to remission of 13.3 days.

In the ISKDC study, prednisone was administered in divided doses. In 1989, the Atlanta group demonstrated that prednisone given at 2 mg/kg as single-morning daily dose resulted in complete remission in all 17 patients with MCD in a mean time of 9.6 days [73]. This study led to many nephrologist to administer prednisone in a single rather than divided doses.

Data on the treatment of initial episode of nephrotic syndrome in adults are scarce. As mentioned above, the studies by Black and Coggins showed that patients treated with prednisone went into remission more rapidly than those receiving placebo The rate of response to prednisone in adults is less than that observed in children. Complete remission is seen in 80 % of adults with MCD, with only 50 % occurring within the first month of therapy and with 10–25 % patients requiring 6–12 weeks of therapy.

The length of induction therapy for the initial episode of nephrotic syndrome has not been defined. It has varied from 4 [2] to 8 weeks [74] (regardless of when remission is induced) to a 3 [75] to 10 [76] day course upon resolution of proteinuria.

In a subsequent study, the Arbeitsgemeinschaft für Pädiatrische Nephrologie (APN) compared the outcome of children with initial episode of nephrotic syndrome treated with the ISKDC protocol (60 mg/m^2/day for 4 weeks followed by 40 mg/m^2/day for 4 weeks) [“short therapy”] versus a 60 mg/m^2/day for 6 weeks followed by 40 mg/m^2/day for 6 weeks regimen [“extended therapy”] [77]. The cumulative rate of patients with sustained remission after 2 years was significantly higher in the extended therapy group compared to the short regimen without increasing the risk for severe steroid side effects. In addition, a frequent relapsing (FR) pattern was observed more often in those patients under the short compared to the extended prednisone regimen (57 % and 29 % respectively). Although tantalizing, these results were not confirmed by the Southwest Pediatric Nephrology Study Group [78]. In their study, the relapse rate was not statistically different between patients receiving the “short” (average of 28 days) versus “extended” (average of 42 days) induction therapy with prednisone.

In our practice, we complete induction therapy with daily prednisone 2 mg/kg/day for 10 days after resolution of proteinuria, a course based on the length of time it takes for serum albumin to normalize. In our experience, this regimen leads to similar rate and timing of remission that than reported by the ISKDC.

The Japan Cooperative Study Group of Kidney Disease in Children compared two regimens of prednisolone [79]. For group A, patients received prednisolone 60 mg/m^2/day for 6 weeks compared to those in group B who received the same dose but for a 4-week duration. At the end of the induction period, both groups had a similar timing of remission (median of 10 days in both groups) and rates of response (34/35 and 36/38 patients respectively). Based on this study, a longer induction did not result in a faster or higher rate of remission.

The addition of cyclosporine to full dose of prednisone in the induction phase did not increase the remission rate nor shorten the timing of remission.

In children, the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines 2012 [80] recommended the induction treatment of the initial episode of nephrotic syndrome in children with daily oral prednisone or prednisolone at 60 mg/

m^2/day or 2 mg/kg/day (maximum 60 mg/day) for 4–6 weeks. In adults, the recommendation is a single dose of prednisone at 1 mg/kg (maximum of 80 mg/day) or alternate-day single dose 2 mg/kg (maximum of 120 mg) until complete remission (minimum of 4 weeks to a maximum of 16 weeks).

In summary, there are several guidelines for MCD treatment but as shown by the Canadian-American, and Italian pediatric nephrologists' surveys [78, 81], the guidelines are not followed**.**

Tapering Therapy

The rationale to taper prednisone is to prevent early relapses and to reduce the risk of side effects such as pseudotumor cerebrii or adrenal insufficiency. During the tapering phase, it is widely accepted that prednisone should be administered on alternate instead of intermittent days (3 consecutive days in a week) [82]. However, there is no agreement on the duration of the tapering phase or the dose of prednisone. Thus, the tapering varies from a 4-week period followed by abrupt discontinuation as proposed by the ISCKD to a slow tapering that ranged from 6 weeks up to 7 months proposed by the Japanese group.

This variability was highlighted by the Southwest Pediatric Nephrology Study in centers in the USA and Canada. This retrospective study compared a "standard" versus an "extended" prednisone tapering which ranged from 4–12 weeks for the former and 6–14 weeks for the latter group. Despite the different length of induction and tapering phase, the number of patients suffering relapse was not significantly higher in the standard compared to the extended group [78]. Bagga et al. [74] showed that an extended prednisolone regimen to 4 months delayed the time to first relapse but there was no statistically difference in the percentage of patients with relapse at 1 year (72.7 % extended vs. 91.3 % standard group). The Japan Cooperative Study Group of Kidney Disease in Children reported a longer sustained remission and fewer cases of frequent relapsing cases in children treated with a 28-week prednisolone course compared to those receiving a 12-week course [79]. The relevance of different tapering regimens in these 2 studies (4 weeks vs. 8 weeks respectively) is limited as the induction regimens also differed among groups in each study (4 weeks vs 8 weeks respectively).

In 2015, a Cochrane systematic review concluded, based on 3 well-designed randomized controlled trial (RCT), that 6-month prednisone course does not reduce the risk of relapse compared with a 2 or 3-month course in children aged 1–17 years at presentation [83]. In our practice, we complete a 6 to 8-week tapering phase starting 10 days upon resolution of proteinuria.

The 2012 KDIGO guidelines for children with idiopathic nephrotic syndrome recommended oral prednisone at 40 mg/m^2 or 1.5 mg/kg on alternate days and continued for 2–5 months with tapering of the dose. The recommendation is more vague for adults, suggesting a slow tapering over a total period of up to 6 months [80].

6.9.2.2 Natural Course of Nephrotic Syndrome After Steroid Treatment

MCD is a relapsing disease in the majority of patients. In 1980, Engle et al. [84] reported a case of late recurrence of nephrotic syndrome in a MCD patient who had been in remission for 19 years after childhood. Subsequently, numerous authors have shown that children with MCD often suffer relapses during adulthood (from 5.5 % to 42 %) [85, 86]. A higher number of relapses per patient per year or a frequent relapsing pattern during childhood were risk factors for relapse during adulthood [85].

The ISKDC provided arbitrary definitions regarding the relapse pattern after a 4-week induction phase followed by a 4-week tapering phase (see Table 6.2)**.** However, these concepts have been widely applied by practitioners despite using different induction and/or tapering regimens. Distinction on the pattern of relapse is often misinterpreted making difficult any comparison of different therapy regimens among studies. One may question whether the significance of a steroid dependent (SD) pattern is similar among different centers given the variability of the tapering schedules. For instance, extended tapering regimens tend to use a very low dose of prednisone towards the end of the tapering period.. Thus, a patient may be labeled as steroid dependent because of a relapse on a much lower dose of steroid as compared to another patient considered steroid dependent according to ISKDC definitions. In summary, longer tapering schedules will lead to a higher number of patients with a "steroid dependent" pattern.

In addition, the original definition of steroid dependence does not take into consideration the role of URI as trigger of nephrotic syndrome. Therefore, it is critical to differentiate the presence of proteinuria associated with viral illness from a full-blown relapse before labeling a patient as steroid-dependent.

This variability is of critical relevance when comparing long-term outcomes of patients labeled as frequent relapser or steroid-dependent to medications including newer drugs such as rituximab and ACTH.

The ISKDC reported the long-term outcome of 363 MCD patients. 93 % of patients achieved remission within 4 weeks. Of these patients, 36 % had no further relapse and 18 % and 39 % had an infrequent and frequent relapse pattern (see Table 6.2 for definitions). Koskimies et al. [87] reported a higher rate of frequent relapse pattern (53 %) among children with idiopathic nephrotic syndrome who responded to an initial course of prednisone (78 of 94 children had confirmed MCD). Of these patients, 64 % became free of relapse after 3 years. 22 % and 24 % of patients had infrequent or no relapses after the initial episode of nephrotic syndrome.

6.9.2.3 Steroid Therapy for Nephrotic Syndrome in Relapse

As for treatment of the initial episode, there is no consensus as to the use of prednisone in the induction and tapering phases. The ISKDC recommended prednisone at 60 mg/m^2/day until response (maximum of 4 weeks) followed by prednisone 40 mg/m^2/day in 3 consecutive days in a week for a total of 4 weeks [2]. Similarly, the APN

[77] and Japanese group [79] treated relapses with prednisone 60 mg/m^2/day until resolution of proteinuria for 3 days, followed by prednisone 40 mg/m^2/48 h for 4 weeks. Bagga et al. [74] suggested prednisolone 2 mg/kg for 2 weeks and then 1.5 mg/kg on alternate days for 4 weeks. As mentioned previously for treatment of an initial episode of MCD, controlled studies have shown that regimens based on a prolonged course or higher cumulative dose of prednisone do not alter the pattern of subsequent relapses.

Resolution of proteinuria is followed by massive diuresis and resolution of edema despite the presence of hypoalbuminemia, which usually normalizes within 7–10 days after remission. In contrast, it may take months for hyperlipidemia to normalize.

6.9.2.4 Therapy for Frequently Relapsing and Steroid Dependent Nephrotic Syndrome in MCD

As shown in Table 6.2, according to the ISKDC, frequent relapsing (FR) is defined by the presence of 2 or more episodes of relapse within 6 months while off steroid therapy for 2 weeks, while steroid dependency (SD) is defined as relapse during the tapering phase or 2 weeks after cessation of steroid therapy.

Historically, studies have included patients without differentiating between frequent relapsing and steroid dependent pattern among these patients. Several drugs have been used to spare the use of prednisone to avoid prolonged exposure to steroids as side effects of high dose steroids for a prolonged period include cataracts, hypertension, stature growth impairment and obesity.

Chlorambucil (0.15 mg/kg/day for 56 days) or cyclophosphamide (2 mg/kg/day for 56 days) used in combination with low dose prednisone have been shown to induce sustained remission (72 % patients at 30 months after cytotoxic drug) in MCD patients with multiple relapses [88]. The ISKDC found fewer episodes of relapse (48 % vs 88 % at 22 months) among those patients with multiple relapses treated with a 42-day course of cyclophosphamide (5 mg/kg/day until induction of cytopenia followed by 1–3 mg/kg/day along with prednisone 10 mg/m^2/day for 10 days) **c**ompared to those on an intermittent dose of prednisone (40 mg/m^2/3 out of 7 days for 6 months) [89]. It is suggested that cyclophosphamide should not be started until the patient has achieved complete remission with prednisone to avoid hemorrhagic cystitis.

Chlorambucil and cyclophosphamide also have serious side effects such neutropenia, hemorrhagic cystitis, late malignancy and gonadal toxicity. Therefore, it is recommended not to exceed a cumulative dose of 168 and 8 mg/kg respectively for these two medications. Second courses of these medications should not be administered.

Prolonged use of cyclophosphamide up to 12 weeks in MCD patients presenting multiple relapses has led to controversial results. The APN reported a higher rate of remission after 2-year follow-up in patients receiving cyclophosphamide for 12 weeks compared to those treated for 8 weeks (67 % vs 22 % respectively) [90]. No such difference was observed by Ueda et al. [91] when compared a 12-week vs

8-week cyclophosphamide regimens in MCD patients after 5 years (24 % vs 25 % respectively). No benefit was observed when cyclophosphamide was administered intravenously at 500 mg/m^2 in a monthly base for 6 months. The use of chlorambucil did not add any benefit to the results observed with cyclophosphamide.

Treatment with cyclosporine results in a similar rate of remission compared to cyclophosphamide and chlorambucil in MCD patients with multiple relapses. However, its long-term efficacy is hampered by the onset of relapse shortly after calcineurin inhibitor (CNI) withdrawal [92]. CNI may be also considered as second agent in MCD patients who relapse frequently. Cyclosporine is recommended at an initial dose of 5 mg/kg/day, and needs to be adjusted to maintain trough serum levels between 100–150 ng/ml. The length of therapy varies from 12 to 24 months. Mild to moderate cyclosporine-associated nephrotoxicity has been reported in up to 1/3 of MCD patients treated with cyclosporine for more than 3 years [93]. Tacrolimus appears to have similar efficacy than cyclosporine but has the benefit of less side effects.

Less data are available on the use of mycophenolate mofetil (MMF) in MCD patients with multiple relapses. Gellermann [94] designed a randomized, open-label, cross-over study to compare the efficacy of MMF vs. cyclosporine in these patients administered during remission (as defined by ISKDC). Patients treated with cyclosporine had a longer free period of relapse during the first year. Only 15 % of patients relapsed during cyclosporine therapy compared to 36 % patients during MMF therapy. However, this difference was not statistically significant. In another randomized control trial [95], 12 children with biopsy proven MCD and multiple relapses were assigned to received MMF or cyclosporine for 1 year. Patients on MMF group had a higher risk for relapse though the difference did not reach statistical significance likely due to the small sample size.

As previously mentioned, the above studies included patients with multiple relapses but they do not differentiate between patients with steroid dependency or those consider to be frequent relapsers. In 1978, we suggested that these patients have a different response to cyclophosphamide [76]. We observed that patients with the frequent relapsing pattern responded very well with a 70 % remission rate after 2 years, while those with steroid dependency did poorly with only 38 % of patients in remission after 3 months of completing therapy. Validating the evaluation of these patients separately, when we combined these two type of patients, the relapse rate was similar to previous studies. These findings were confirmed by APN study in 1983 using the same therapeutic schedule [88]. Therefore, we do not recommend the use of either cyclophosphamide or chlorambucil in the steroid dependent MCD patient.

The term steroid dependent nephrotic syndrome needs also to be strictly defined. Relapse while on prednisone should be the sine qua non of the term. As mentioned above, the interpretation of studies comparing outcomes and response to therapy is hampered by the use of prednisone tapering of variable length and dose. Thus, the minimal amount of prednisone to consider a patient as SD may vary from patient to patient but should not be less than 10 mg/day or 20 mg every other day. One alternative approach for the Steroid Dependent patient is to continue with steroids at the lowest dose that keep patient in remission, given on alternate days. A dose of prednisone less than 0.5 mg/kg/day should not interfere with statural growth.

6.9.2.5 Other Medications in MCD

Mizoribine

One RCT including 197 children with frequent relapse showed no different rate of relapse among treatment and placebo groups [96]. Thus, mizoribine is currently not recommended in nephrotic syndrome.

Azathioprine

The ISKDC in a controlled, randomized study concluded that azathioprine has no effect in the relapse rate of children with steroid-sensitive nephrotic syndrome [97].

Levamisole

Levamisole anthelminthic agent that has been shown to reduce the risk of relapse in patient with frequent relapsing and steroid dependent NS in single center randomized controlled trials [98, 99]. Despite these favorable results and an overall safe profile, the use of levamisole is hampered by the lack of a large-multicenter RCT confirming its efficacy and its unavailability in many countries including USA.

Adrenocorticotrophic Hormone (ACTH)

The use of ACTH was first reported in nephrotic syndrome in 1950 [100]. It was found that most of the patients treated with ACTH experienced massive diuresis with about half of them having clinical remission for at least 3 months. For years, ACTH was the first line agent to treat nephrotic syndrome and was approved the Food and Drug Administration (FDA) as therapy for nephrotic syndrome, alongside with steroids and cyclophosphamide. However, the introduction of prednisone for MCD treatment with its benefits of low cost, similar efficacy, and ease of administration halted the use of ACTH in MCD.

The majority of recent data on ACTH in nephrotic syndrome focus on patients with membranous nephropathy. Furthermore, in the passive Heyman nephritis model, ACTH seems to exert its antiproteinuric effect via podocyte MC1R receptor [101].

There is very limited data on ACTH in MCD. In a retrospective case series [102], one MCD patient, previously treated with steroids, mycophenolate and calcineurin inhibitors; received 80 subcutaneous units of ACTH gel twice weekly during 4 months without observing any reduction in proteinuria. In a prospective open-label study [103], two MCD patients resistant to other immunosuppressive therapy (steroids, mycophenolate, tacrolimus and rituximab in 1 patient) were treated with ACTH for 24 weeks. One patient remained nephrotic at the end of the trial and the other MCD patient underwent partial remission during ACTH trial but relapsed shortly after completion of ACTH therapy. Thus, there is no clinical evidence to

support the use of ACTH in MCD at this time. There is an ongoing randomized controlled trial to assess the efficacy and safety of ACTH versus placebo in children with frequent relapsing and steroid dependent syndrome.

Rituximab

A large number of observational studies have been published in the last decade on the use of rituximab in nephrotic syndrome induced by a variety of glomerulopathies including MCD. Only recently, however, have a few randomized controlled studies been published.

Despite a decade of clinical experience with rituximab in MCD, the mechanism(s) by which it may induce remission is unclear. Since the putative circulating factor likely to cause proteinuria in MCD has been assumed to be a cytokine released by abnormal T cells and rituximab acts on B cells, it has been suggested that B cells have a pathogenic regulatory role on T cells in MCD. This pathogenic role could be "reversed" by rituximab. However, the evidence to support a role of B cell on T cells in MCD is lacking [104]. More recently, rituximab was found to bind sphingomyelin phosphodiesterase acid-like 3 b protein (SMPDL-3b) expressed in podocytes, suggesting that rituximab may play a direct, rather than immune-mediated, role on podocytes. SMPDL-3b expression is decreased in kidney tissue from FSGS patients who had recurrence of proteinuria as well as in cultured podocytes treated with sera from FSGS patients with recurrence [105]. It has been hypothesized that decreased expression of SMPDL-3b may lead to decreased acid-sphingomyelinase (ASMase) activity in the raft microdomains which in turn could contribute to actin cytoskeleton remodeling by a mechanism(s) yet to be determined. The above experimental data supports that rituximab may target the podocyte in FSGS but its role in MCD has not been established.

Since 2011, four randomized controlled trials (3 of which came from the same group) were designed to determine the efficacy and safety of rituximab in nephrotic syndrome. Magnasco et al. [106] found that rituximab failed to reduce proteinuria at 3 months in patients with steroid resistant nephrotic syndrome compared to those not receiving rituximab. Only 7 MCD of 31 patients were included in this study. Ravani et al. [107] suggested that rituximab and lower doses of prednisone and calcineurin inhibitors were non-inferior to standard doses of steroids in patients with steroid-dependent nephrotic syndrome. The authors reported that patients in the rituximab group had less proteinuria and a fewer number of relapses at 3 months. However, the relevance of this study to support the use of rituximab in MCD is limited by several facts: (1) it is unclear if the power analysis is based on a non-inferiority-based model as sample size for the 4 groups included in the study are not specified, (2) only 6/27 and 13/27 patients had biopsy-proven MCD in the control and intervention group (rituximab) respectively, (3) patients with high dose steroid dependency (0.7 mg/kg/day) were excluded, (4) it is unclear how many of the patient were in relapse or remission at the time of randomization, (5) data interpretation after 3-month follow-up is limited since many of patients from the control group were switched to the treatment group at 3 months, (6) 75 % of patients in the rituximab group relapsed at 1-year follow-up.

The same authors [108] recently reported a non-inferiority randomized controlled trial including children with steroid-dependent nephrotic syndrome in remission. The intervention group received one dose of rituximab at randomization, followed by steroid tapering after 1 month, similarly to the control group. The primary outcome of the study was the degree of proteinuria within 3 months after randomization. Proteinuria was lower at 3 months in the rituximab group although it did not reach statistical significance. Interestingly, only 34 % of patients in the rituximab group relapsed at 1 year follow-up in contrast with the 75 % previously reported by the same group. Of interest, patients on this study received a single dose of rituximab and had steroid dependency at higher dose prednisone (>0.7 mg/kg/day) compared to the already cited previous study in which patients received 1 or 2 doses of rituximab and had a lower degree of steroid dependency.

Finally, Iijima et al. [109] found that patients with "complicated" SD or FR nephrotic syndrome receiving weekly rituximab for 4 weeks after remission had been induced had a significantly longer relapse-free period and fewer relapses compared to those patients receiving placebo (median time to first relapse 267 versus 101 days and 1.54 versus 4.17 relapses/person/year respectively). Treatment failure defined as relapse by 13 weeks after randomization, or steroid resistance between weeks 1 and 53 or FR/SD pattern between weeks 13 and 53, was more frequent in control patients (20/23) compared to those receiving rituximab (10/20). No relapses were reported in the rituximab group during the period of B-cell depletion. The authors concluded that rituximab should be considered as an effective treatment for children with complicated FR/SD nephrotic syndrome during remission. However, such a conclusion may be premature based on the results of this study: (1) Based on authors' definition for treatment failure, 50 % of patients in rituximab group failed therapy; (2) most/all patients relapsed in both groups (17/20 vs. 23/23, rituximab vs. control respectively) by the end of the study; (3) the patient population in this study was heterogeneous in terms of being frequent relapses or steroid dependent and in terms of their immunosuppressive regimens and patients included in this study did not follow the same prednisone tapering used by ISCKD. In addition, the authors presented the long-term outcome of all patients as a group but did not differentiate among those patients presenting with a frequent relapsing versus steroid dependent pattern at the beginning of study. Thus, it is unclear whether FR and SD patients have a similar response to rituximab. In this study, more than 70 % of the included patients had steroid side effects such as hypertension, short stature, diabetes, glaucoma, cataract, obesity and osteoporosis. Patients in the rituximab group received a significantly lower cumulative prednisone dose during 1-year follow-up compared to those in the control group. However, no statistically difference in height-for-age Z score and blood pressure were found among both groups of patients at the end of the study.

In summary, the current evidence supporting a role of rituximab in the treatment of MCD is not conclusive. Randomized controlled trials with a larger and well defined cohort of patients and long-term follow-up (>1 year) are needed to assess the efficacy and safety of rituximab before its widespread use in MCD.

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Chapter 7
Focal Segmental Glomerulosclerosis and Its Pathophysiology

James Dylewski and Judith Blaine

Abbreviations

ACEi	Angiotensin converting enzyme inhibitor
APOL1	Apolipoprotein L1
ARB	Angiotensin receptor blocker
BP	Blood pressure
CLC-1	Cardiotrophin-like cytokine 1
CNI	Calcineurin inhibitor
COQ	Coenzyme Q
ESRD	End stage renal disease
FSGS	Focal segmental glomerulosclerosis
HAN	Heroin associated nephropathy
HIVAN	Human immunodeficiency virus associated nephropathy
KDIGO	Kidney disease improving global outcomes
MAP	Mean arterial pressure
MMF	Mycophenolate mofetil
mTOR	Mechanistic target of rapamycin
RAAS	Renin angiotensin aldosterone system
suPAR	Soluble urokinase plasminogen activator receptor
TGF-β	Transformimg growth factor beta
TNFα	Tumor necrosis factor alpha
TRPC6	Transient receptor potential cation channel 6

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7.1 Introduction

Focal segmental glomerulosclerosis (FSGS) is a morphological finding on light microscopy of renal tissue that has become synonymous with a progressive proteinuric kidney disease. As the name may suggest, FSGS is when some (or focal) glomeruli have sclerosis (or scarring) in segments of the glomerular tuft. This pattern is typically seen early in the disease process but can become more extensive as the disease evolves [1, 2]. This pattern of scarring is not unique to a singular cause and is applied to kidney diseases caused by genetic mutations, viral infections, medications, toxins, reduced renal mass, or a "permeability factor". Though the causes of this disease may be quite diverse, injury afflicted on the podocyte is what leads to scarring of the glomerulus and proteinuria [1].

7.2 Primary FSGS

Typically when the term "primary" is applied to other disease states, such as primary immune deficiency, it refers to an in-born deficiency or mutation. Conversely, "Primary FSGS" is usually only applied to the idiopathic form of the disease. In fact, leading experts in the area of FSGS typically classify genetic causes as a secondary form of FSGS [1–3]. Given the difference between FSGS and other disease classification standards, it can create confusion and debate among those not familiar with the terminology by which FSGS is classified.

Idiopathic FSGS, as the name suggests, is caused by an entity not fully understood at present. It has been suggested that idiopathic FSGS shares some commonality with minimal change disease. Proponents of such an association cite the fact that early histologic changes in idiopathic FSGS are similar to minimal change disease. There are documented cases of patients presenting with nephrotic syndrome and initial biopsies which reveal minimal change disease histology with subsequent biopsies revealing progression to FSGS [1]. Challengers to this theory suggest these observations maybe a result of sampling error given that the changes of FSGS, by definition, only affect some glomeruli and could easily be missed given the limited amount of tissue obtained on a normal biopsy. Either way, FSGS and minimal change disease are similar in that both result in a proteinuric renal disease and are primarily diseases of podocytes. In addition, the causes of both minimal change disease and FSGS are not fully understood.

Several observations about idiopathic FSGS have generated a possible theory for its cause [4]. First, proteinuria and recurrent disease can develop within minutes after transplantation in recipients with idiopathic FSGS [5–7]. Second, when serum from idiopathic FSGS patients was injected into rats, the previously healthy rats developed proteinuria [8, 9]. Third, there are reports of pregnant women with idiopathic FSGS who delivered children that had transient proteinuria immediately after birth [10]. Fourth, there is improvement in the amount of proteinuria in patients with recurrent FSGS who were treated with plasma exchange or protein absorption

[11, 12]. Finally, when kidneys with recurrent FSGS are re-transplanted into patients with kidney disease other than FSGS, the proteinuria and histological changes resolve [13, 14]. These observations suggest the cause of the disease is intrinsic to the individuals' blood and not the kidney itself. Therefore, it has been theorized that the disease is caused by a circulating permeability factor that attacks and damages the podocytes. The exact identity of this permeability factor has yet to be delineated, although a few proteins have been identified as potential sources.

Cardiotrophin-like cytokine 1 (CLC-1), is part of the IL-6 family and is believed to be produced by CD34+ stem cells and is inactivated by galactose *in vitro* [1, 15]. It has been observed that patients with idiopathic FSGS have an overabundance of CLC-1 in their sera when compared to those without the disease [16]. Podocytes have a receptor for CLC-1 that is noted to be upregulated in patient with recurrent FSGS and in patients with idiopathic FSGS when there is an overabundance of this cytokine in the sera [17]. McCarthy et al. also report that CLC-1 can induce proteinuria in experimental models and decrease podocyte expression of nephrin, an important protein that is needed for maintenance of the slit diaphragm. Additionally, anti-CLC-1 antibody when used with both isolated rat glomeruli and FSGS patients' sera has been shown to mitigate the permeability of albumin through the glomeruli [16]. However, the role of CLC-1 *in vivo* is not as well demonstrated. Additionally, the use of galactose infusions, which should inactivate CLC-1, has failed to consistently prevent recurrent FSGS clinically [3].

Another proposed factor is soluble urokinase plasminogen activator receptor (suPAR). Urokinase plasminogen receptor (uPAR) is a receptor that can be found on podocytes and is involved with cell migration and slit diaphragm maintenance by forming signaling complexes with cell membrane proteins such as αvβ3 integrin [18]. The soluble form, suPAR, can be released from the plasma membrane by leukocytes and podocytes, and has been demonstrated to be increased in FSGS [19]. When suPAR undergoes cleavage and glycosylation, it is believed to make an isoform that would fit the characteristic of the theorized causative factor for FSGS [4]. It has been reported that suPAR levels are higher in patients with FSGS compared to other diseases with similar proteinuria and, in cohort studies, the majority of FSGS patients have elevated suPAR levels in the serum [1, 19]. SuPAR has also been noted to be elevated in patient with recurrent FSGS after transplantation [20]. Additionally, in one study, when mice were given injections of suPAR they developed glomerular deposits of suPAR that were associated with podocyte effacement, proteinuria, renal dysfunction, and glomerular damage [4, 18, 19]. Finally, observation studies have suggested that patients treated with plasmapheresis that resulted in lower suPAR levels were associated with reduced proteinuria and remission [4]. However, it has been noted that there was no significant difference in suPAR levels between FSGS and non-FSGS patients when matched for estimated renal function [21] and that suPAR can be elevated in FSGS caused by genetic mutations [22]. Furthermore, pre-transplant serum suPAR levels did not correlate with recurrent disease [23]. Moreover, Cathelin et al. demonstrated that suPAR glomerular deposits do not necessarily result in proteinuria and abnormal podocyte features [24]. For these reasons, some have proposed elevated suPAR levels may be more of a correlation, rather than a causative agent, in primary FSGS but more studies are needed.

7.3 Secondary FSGS

As mentioned above, FSGS histology represents a common end result for a multitude of causes. Secondary causes include etiologies such as genetic disorders, viral infections, drug related, or the result of an adaptive change.

7.3.1 Genetics

In recent years, several genetic mutations have been determined to be associated with renal dysfunction and histologic morphology of FSGS (see Table 7.1). Each of the mutations result in abnormalities in one of six broad categories: (1) slit-diaphragm related molecules, (2) podocyte actin cytoskeleton, (3) podocyte signaling, (4) podocyte gene transcription, (5) molecules for adhesion or extracellular matrix, or (6) mitochondrial DNA or COQ synthesis (Table 7.1 [3, 25–29]). Although these mutation share commonality in their renal manifestations, these mutations also occur in other organ systems and can be associated with a wide array of other clinical manifestations.

A particular area of interest recently is the association with FSGS, hypertensive nephrosclerosis, and HIV-associated nephropathy in African descendants and the roles that apolipoprotein L1 (APOL1) and myosin heavy chain 9 (MYH9) play in these diseases. Kao et al. and Kopp et al. noted in 2008 an association between variants in myosin heavy chain 9 (*MYH9*) gene on chromosome 22 and FSGS in African Americans [30, 31]. However, a causative variant for the *MYH9* associated FSGS could not be identified. With additional research a variant in the neighboring *APOL1* gene was identified and determined to have a much stronger association with the disease [32, 33]. *APOL1* is a gene encoding for apolipoprotein L1 (also called APOL1), a lipoprotein that is part of a larger APOL family involved in innate immunity [34]. The *APOL1* gene has 2 variant alleles, G1 and G2, which represent a missense and a deletion mutation, respectively, compared to the non-pathological allele, G0. The G1 and G2 variants are recessive and individuals have an increased likelihood of developing kidney disease if two risk alleles are present (i.e. homozygous G1/G1, homozygous G2/G2, or heterozygous G1/G2) [34]. Twelve to thirteen percent of African Americans carry two risk alleles [35]. Interestingly, APOL1 is not physiologically necessary and is absent in other primates and certain populations of humans [34, 36]. However, it has been shown that APOL1 can insert itself as a pore into the lysosome of *Trypanosoma* spp., a genus of parasite associated with the deadly disease African sleeping sickness, leading to swelling and parasite lysis [37, 38]. The G1 and G2 variants of the APOL1 seem to provide broader protections to certain strains of the *Trypansoma* spp. [39]. This likely provided a survival benefit to the ancestors of its carriers and thus explains its high prevalence today.

The mechanism by which APOL1 leads to kidney disease is incompletely understood. Lan et al. showed that APOL1 can induce podocyte injury by increasing

Table 7.1 Genetic mutations associated with development of FSGS [13, 24–28]

Gene	Product affected	Site of abnormality	Inheritance
NPHS1	Nephrin	Slit diaphragm	AR
NPHS2	Podocin	Slit diaphragm	AR
CD2AP	CD2-associated protein	Slit diaphragm	AR
ACTN4	Alpha actinin-4	Podocyte cytoskeleton	AD
MYO1E	Non-muscle myosin-IE	Podocyte cytoskelton	AR
MYH9	Non-muscle myosin-IIA	Podocyte cytoskeleton	AD
INF2	Inverted formin-2	Actin organization	AD
ARHGDIA	Rho GDP-dissociation inhibitor 1	Actin dynamics, signaling with Rho GTPase	AD
TRPC6	Transient receptor protein calcium channel 6	Podocyte ability to react to stimuli	AD
PTPRO	Receptor tupe tyrosine-protein phosphatase O	Podocyte to podocyte signaling	AR
PLCε1/NPHS3	PLCε1	Podocyte signaling and development	AR
SCARB	Scavenger receptor class B member 2	Putative lysosomal receptor	AR
LAMB2	Laminin beta 2	GBM to actin cytoskeleton interaction	AR
CD151	Tetraspanin	GBM and podocyte interaction	AR
WT-1	Wilm's tumor protein 1	Podocyte development	AD
LMX1b	LIM homebox transcription factor 1β	Podocyte and GBM development	AD
ITGB4	B4-integrin	Adhesion to cell-matrix	AR
SMARCAL1	SNF-related matrix associated actin-dependent regulator of chromatin subfamily A-like protein 1	Gene transcription	AR
MTTL 1	Mitochondrially encoded tRNA leuine 1	Mitochondrial tRNA	Maternal
COQ2	Polyprenyltransferase	Coenzyme Q10 biosynthesis	AR
COQ6	Ubiquinone biosynthesis monooxygenase COQ6	Ubiquinone biosynthesis	AR
PDSS2 [24]	Decaprenyl diphosphate synthase subunit 2	Decaprenyl tail of coenzyme Q10 production	AR
ADCK4 [25]	AarF domain-containing protein kinase 4	Coenzyme Q10 modulation	AR

AR = autosomal recessive; AD = autosomal dominant

lysosomal membrane permeability leading to swelling in a viral infection model and that the podocyte injury was particularly dramatic in HIV infected cells with the G1 or G2 variants [40]. Additionally, these researchers showed that media from the G1 and G2 variants can induce injury in non-infected podocytes, suggesting a secreted substance from the G1 and G2 variants as an additional means of injury [40].

Despite a seemingly strong association with FSGS and the variant alleles of APOL1, individuals with two APOL1 risk alleles only have a 4 % estimated lifetime risk of developing CKD. However, in the setting of two risk alleles and untreated HIV, the lifetime risk increases by as much as 50 % [35]. Based on these findings, some have come to suggest an additional insult (or insults) is necessary for developing chronic kidney disease. Some have speculated that the coexistence of sickle cell trait may be one of the factors that lead to chronic kidney disease in this population but more studies are needed [41].

7.3.2 Drugs

In the 1970's, Rao et al. noted a correlation with heroin addiction and proteinuric kidney disease with FSGS morphology on kidney biopsy. This prompted the term heroin-associated nephropathy or HAN [42]. In present times however, HAN has become uncommon, presumably due to the improved purity of heroin [2, 43]. It has been theorized that since HAN was more commonly observed in African American individuals, it may have some relation to APOL1 risk-variants [44]. Cases of FSGS occurring in bodybuilders who had used anabolic steroids for a prolonged period have also been reported and may be related to increased muscle mass leading to adaptive glomerular changes (which will be discussed later) and/or a toxic effect of the steroids themselves [45].

Non-illicit drugs have been associated with FSGS as well. Pamidronate, a bisphosphate that works on osteoclasts through multiple mechanisms including modulation of the actin cytoskeleton, may also affect the podocyte actin cytoskeleton given its associated with the collapsing variant of FSGS [2]. Interferon alfa, beta, and gamma all have effects on podocytes and when used exogenously for treatment, can induce FSGS as well. A nephrotic syndrome associated with lithium has been reported in case studies. Although the histology associated with lithium use is most often consistent with minimal change disease, FSGS has also been reported [46].

In kidney transplants, sirolimus (rapamycin), an mTOR inhibitor, has been associated with proteinuria and an FSGS pattern. Vollenbröker et al. showed that sirolimus can alter the expression of slit diaphragm proteins nephrin, TRPC6, and other proteins which are needed for podocyte adhesion and motility [47]. The impaired expression of these proteins has been associated with a FSGS pattern of injury (see above under genetic causes). Calcineurin inhibitors have been cited as having an association with collapsing FSGS in kidney allografts, possibly through their vasoconstrictive effects [2]. However, the occurrence of collapsing FSGS in kidney allografts is relatively uncommon despite the wide use of calcineurin inhibitors.

Additionally, the case series of collapsing FSGS in transplant recipients suggest a diverse array of causes, including immune complex deposits, and the characteristics of the donors, who might have had a predisposing risk factor such as *APOL1* risk alleles [48–50], were not mentioned.

7.3.3 Infectious

HIV-associated nephropathy (HIVAN) has been associated with collapsing FSGS, particularly among African American individuals with two *APOL1* risk alleles [35]. HIV RNA and circular viral DNA have been detected in renal glomerular and tubular epithelial cells from kidney biopsies [51] but HIV is unable to replicate once internalized by the podocyte [52]. In mouse models, expression of certain HIV genes can induce podocyte and renal tubular epithelial cells to dedifferentiate leading to proteinuria and collapsing FSGS histology [53–55].

Infections with cytomegalovirus (CMV), Epstein-Barr virus (EBV) and parvovirus B19 have also been reported to cause collapsing FSGS. However, they are less common and their mechanism of injury is not as well characterized [56].

7.3.4 Adaptive Response

Focal segmental glomerulosclerosis can develop over time as a result of reduced renal mass or hemodynamic changes that lead to a maladaptive scarring (for causes see Table 7.2 [1]). The mechanism for the disease initially results from increased glomerular capillary pressure.

In animal models, when a significant portion of kidney is removed there is a vasodilation in both the afferent and efferent arterioles causing increased glomerular blood flow [57, 58]. Since the reduction in vascular resistance is disproportionally more in the afferent arterioles, inter-glomerular hypertension develops due to the rise in glomerular hydrostatic pressure [2]. This results in each remaining

Table 7.2 Etiologies for adaptive causes of FSGS

Reduced renal mass	Hemodynamic stress
Very low birth weight	Prolonged hypertension
Oligomeganephronia	Vaso-occlusive disease
Unilateral renal agenesis	Atheroembolic disease
Surgical nephrectomy	Obesity-related
Surgical excision or ablation	Increased lean body mass
Cortical necrosis	Cyanotic heart disease
Unilateral renal atrophy	Sickle cell disease
Reflux nephropathy	

nephron hyper-filtrating and thus compensating in an attempt to maintain overall glomerular filtration rate [58]. The glomerular hypertension leads to an increase in glomerular volume which is not accompanied by any increase in podocyte number. The podocytes, which become strained since they cannot readily divide to cover the expanded area, hypertrophy and detach from the basement membrane resulting in proteinuria and sclerosis (see below under common pathway for more details) [58].

In cases where there is increased hemodynamic stress (see Table 7.2), the mechanism is similar in that glomerular hypertension develops leading to glomerular hypertrophy, podocyte defects or detachment, and sclerosis. Both morbid obesity and an elevated lean body mass have been reported to cause an FSGS pattern of injury secondary to glomerular hypertension [1].

It is important to note that other diseases that affect the podocytes and glomerulus can have a focal and segmental pattern of glomerulosclerosis. Diabetic nephropathy, IgA nephropathy, membranoproliferative glomerulonephritis, post-infectious glomerulonephritis, pauci-immune glomerulonephritis, tubulointerstitial diseases, thrombotic microangiopathy, Alport's disease, and hypertensive nephrosclerosis have all been noted to have features that can resemble FSGS depending on the stage [3, 59, 60].

7.4 Pathology

7.4.1 Common Pathway Leading to Podocyte Injury

As previously mentioned, the causes of FSGS may be diverse, but it is believed they share a common pathway that leads to the characteristic pattern of injury. Podocytes have an actin cytoskeleton which enables podocytes to move their foot processes in response to mechanical or chemical stimuli. In FSGS, an insult or a genetic defects causes the podocytes to detach its foot processes from the glomerular basement membrane (also known as foot process effacement). With the effacement of the foot processes, the glomerular filtration barrier is compromised leading to loss of selectivity and proteinuria [61]. If the injury or abnormality persists, it leads to further effacement until the podocyte separates from the basement membrane and subsequently dies. The foot processes of podocytes not only act as a filter but also interdigitate with other podocytes forming a complex network for signaling. As an individual podocyte dies, the remaining podocytes are affected by its loss. The surviving podocytes hypertrophy to cover the denuded area leading to further foot process effacement and stripping of the basement membrane [62]. Additionally, death of a single podocyte may promote death of neighboring podocytes due to exposure to toxic factors (such as angiotensin II or tumor growth factor β) which are released by the dying podocyte, the loss of supporting factor previously produced by the podocyte (such as nephrin or vascular endothelial growth factor), increased mechanical strain, or a combination of all of these.[2, 63–66]. This pattern of propagation has been describe as a "domino-like effect" because the death of one podocyte seems to lead to death of additional podocytes [2].

The parietal epithelial cells from Bowman's capsule migrate to the stripped basement membrane as a possible reparative mechanism [67, 68]. However, the interaction of the basement membrane and parietal epithelial cells can create adhesions between the capsule and the basement membrane. These adhesions can then progress to sclerosis [68, 69]. Since this injury and apoptosis are localized to adjacent cells, it is not uncommon for lesions to appear segmental early in the disease course and then become more widespread as the disease progresses. Wharram et al. demonstrated in transgenic rats that once >40 % of podocytes are involved, the hallmark clinical features of severe proteinuria and renal insufficiency develop. However, they noted that significant proteinuria can develop with fewer podocytes being involved [70].

As more podocytes are afflicted, the sclerosis spreads through the glomerulus resulting in more capsular adhesions. These adhesions are believed to alter glomerular filtrate which may cause tubular simplification, interstitial injury, and eventual fibrosis [68]. These adhesions may also act as a bridge for peri-glomerular fibroblasts to migrate into the glomerular tuft and cause more glomerulosclerosis [68].

As more nephrons become affected, the renin-angiotensin system becomes more active. This leads to formation of angiotensin II which can promote podocyte apoptosis [71]. As more podocytes die, the remaining podocytes have increased protein uptake. The excessive protein uptake activates intracellular transforming growth factor-beta (TGFβ) [72] which can lead to endoplasmic reticulum stress, changing of the cytoskeleton, dedifferentiation, and apoptosis [73].

7.5 Histological Type

In 2004, Drs. D'Agati, Fogo, Bruijn, and Jennette proposed classifying FSGS into 5 categories (Cellular, Collapsing, Classic or not otherwise specified/NOS, Perihilar and Tip) based on the appearance on light microscopy to help with determining the potential etiology of the disease as well as improve accuracy of the diagnosis [74]. Though not universally accepted as definitive for diagnosis, certain variants can be associated with specific etiologies of FSGS. It is important to note that other primary glomerular disease such as diabetic glomerulosclerosis and chronic glomerulonephritis can have similar features to FSGS to the untrained eye. Therefore, it is important to take in all aspects of the biopsy and the clinical history before ascribing this pattern of injury to a specific entity. Please see Fig. 7.1 for histological images of each subtype and Table 7.3 for the details about the clinical features and potential clinic correlation of each subtype.

Immunofluorescence staining of FSGS normally shows coarse segmental IgM and C3 deposits at the sites of hylanosis, although deposits of C1 can also occur [2, 75]. It is important to stain for IgA since advanced IgA nephropathy can appear like FSGS with the only difference being the finding of mesangial IgA deposits in IgA nephropathy [76].

Electron microscopy plays an important role in the diagnosis of focal segmental glomerulosclerosis. Not only does it help rule out advanced depositional diseases

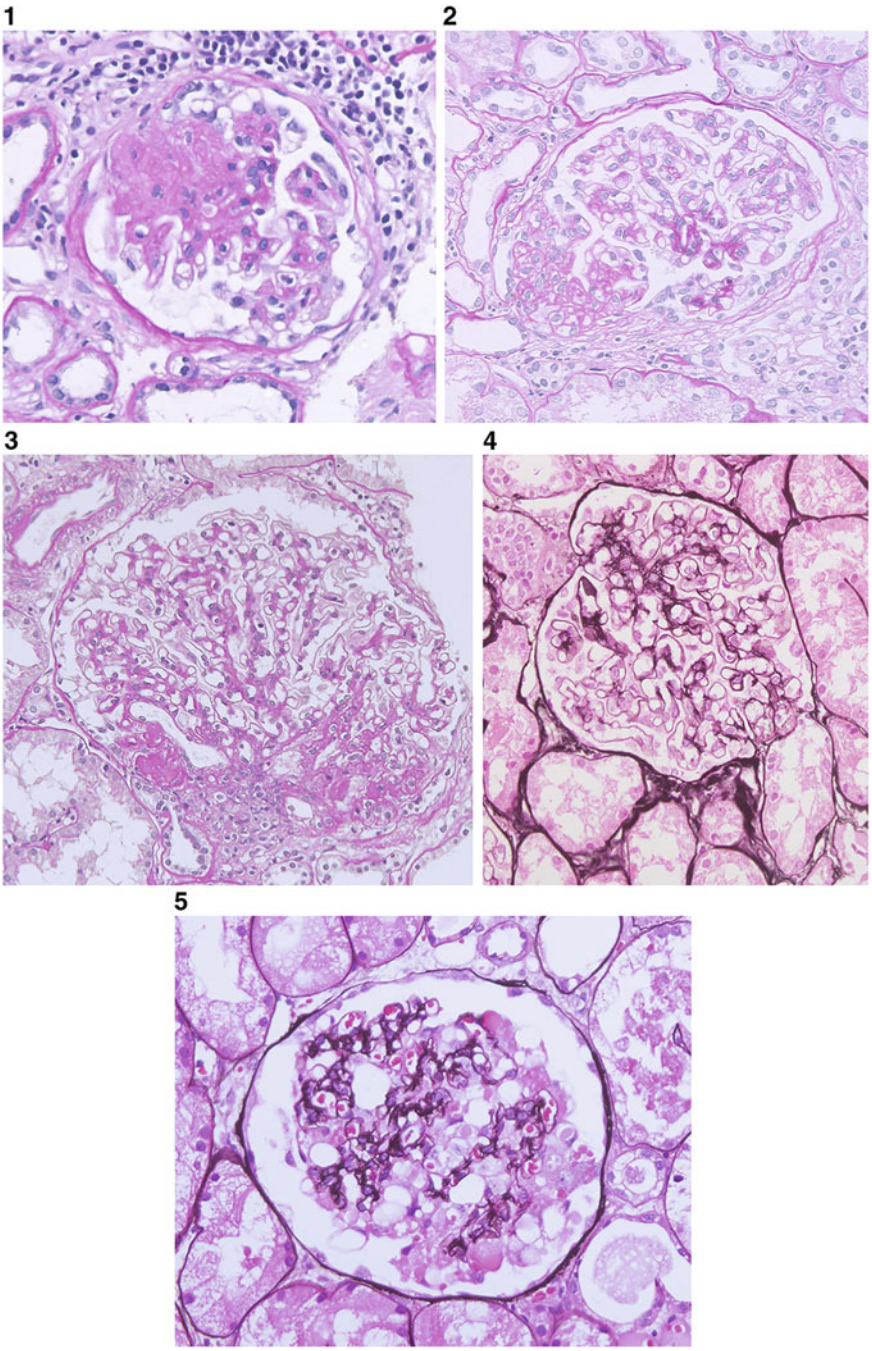

Fig. 7.1 Histologic variants of FSGS. (**1**) FSGS, NOS, PAS stain; (**2**) FSGS cellular variant, PAS stain; (**3**) FSGS, hilar variant, PAS stain; (**4**) FSGS, tip variant, Jones' silver stain; (**5**) FSGS, collapsing variant, Jones silver stain. All images are 400× magnification and are courtesy of Agnes B. Fogo, MD, Department of Pathology, Microbiology and Immunology, Vanderbilt University

Table 7.3 Classification, histology, and clinical features of variants of focal segmental glomerulosclerosis [2, 13, 52–58, 60]

Variant [74]	Major features [3, 73]	Clinical characteristics [2]	Associated causes [2]	Prognosis [75–78]
Cellular	– Increased cellularity of segments of endocapillary loops (endocapillary proliferation) resulting in collapsed lumina – Can have hylanosis	– Rarest variant – Usually presents with nephrotic syndrome – Believed to be an early stage in sclerosis formation	– Most commonly associated with the idiopathic cause but can be seen with secondary causes	– Fair – Remission ≥ progression to ESRD
Collapsing	– Shrunk glomerular tuft in relation to bowman's capsule – Podocyte hyperplasia and hypertrophy – *No* increased intracapillary cellularity or matrix – Can be segmental or global	– Highest prevalence in Black race – Nephrotic syndrome with massive proteinuria	– Can be seen with idiopathic – Most often related to viral, medication or vascular disease	– Very poor – Remission ≪ progression to ESRD
"Classic" or not otherwise specified (NOS)	– Segmental glomerular capillary collapse – Increased matrix –All other types must be excluded	– Most common variant – Variable level of proteinuria	– Can be seen with all etiologies of disease – Other variants and diseases can evolve into this types over time	– Fair to poor – Remission ≤ progression to ESRD
Perihilar	– Sclerosis and hylaninosis near glomerular cleft of vessels (hilum)	– Typically, sub-nephrotic proteinuria	– Often result of adaptive changes (i.e. reduced renal mass, increased body mass)	– Poor – Remission < progression to ESRD
Tip	– Limited to outer 25 % of glomerular tuft – Must have adhesion or podocytes attached to parietal or tubular cells – Must *not* have perihilar sclerosis	– More common in Caucasians – Abrupt onset of nephrotic syndrome	– Most often seen in idiopathic disease	– Best – Remission ≫ progression to ESRD

but it can also suggest the etiology of the disease. Deegens et al. have proposed that foot process width greater than 1500 nm is sensitive and specific for primary FSGS [77]. Though some have suggested that biopsies with a higher percentage of foot process effacement are associated with primary/idiopathic FSGS, studies looking at percentage of foot process effacement showed no statistical difference between those with primary/idiopathic FSGS and those with a secondary form. However, these studies were small and likely underpowered for this statistical analysis [75, 77]. Therefore, the percentage of foot process effacement can be helpful when used in conjunction with other clinical and histologic data [4].

7.6 Clinical Features

7.6.1 Epidemiology

Several studies have suggested an increased incidence of focal segmental glomerulosclerosis in both children and adults around the world over the last few decades [1]. Some of these studies have shown a steady increase in the incidence of FSGS since the 1970's and that FSGS has now become the leading cause for primary glomerulonephritis in countries like Brazil [78–82]. However, studies out of the UK and Korea suggest a relative constant incidence of FSGS during a similar period of time [83, 84]. The reason for the increased incidence among some populations, while not among others, has yet to be explained.

In the US, the rate of ESRD from FSGS has increased by 11 fold over a 21 year period, especially among black individuals [85]. Even among a predominately Caucasian population, there was a 13-fold increase in biopsies with FSGS between 1974 and 2003 [86]. Some have speculated that part of the increase may be related to changes in disease classification and biopsy practices but it is generally accepted the prevalence of FSGS is on the rise. Primary FSGS is more common among males, who also have a 1.5 to 2-fold higher risk of progressing to ESRD, compared to females [85]. Black individuals have a higher incidence of FSGS compared to Caucasians, both in childhood and adulthood [1].

7.6.2 Presentation

Individuals with FSGS can have a mixture of presenting features. Classically, primary or idiopathic FSGS in adults present as having nephrotic syndrome (defined as proteinuria > 3–3.5 g/day, serum albumin < 3.5 g/dl, and peripheral edema). Hypertension, microscopic hematuria, and elevated serum creatinine can also occur but these have been reported to occur less frequently [59, 87]. However, depending on the underlying cause, severity, and the stage of the disease, individuals can present with sub-nephrotic proteinuria, preserved serum albumin, minimal to no

peripheral edema, and normal serum creatinine [1]. Some have suggested that the lack of nephrotic syndrome and sub-nephrotic range proteinuria is more indicative of a secondary form of FSGS [4, 59].

7.6.3 Prognosis

The prognosis of FSGS is quite variable which is likely related to the diversity of causes. In general, compared to other glomerular diseases, such as minimal change disease, the rate of spontaneous remission is uncommon (approximately 5–23 % depending on the study) [88, 89]. There have been a few features that have been suggestive of overall risk of developing end-stage renal disease (ESRD). One factor that has been associated with worse outcomes over several decades is the degree of proteinuria [88–93]. Comparatively, patients with sub-nephrotic range proteinuria have less than a 15 % chance of progression to ESRD at 10 years while nephrotic patients have an approximately 50 % chance of ESRD at 5–10 years [59, 88]. An even worse outcome was observed in individuals with "massive proteinuria" (defined as >10–14 g of protein/day). Patients with massive proteinuria progressed to ESRD within 2–3 years on average [59, 93]. An important caveat to proteinuria as a prognostic indicator is the individual's response to therapy. In the study by Rydel et al., patients with nephrotic range proteinuria who obtained a complete remission (defined as less than or equal to 0.25 g of proteinuria/day) or a partial remission (defined as 0.26–2.5 g of proteinuria/day) had approximately the same rate of renal survival as those with sub-nephrotic proteinuria at 10 years. Additionally, nephrotic patients who either received no treatment or did not respond to treatment had comparable renal outcomes at 10 years [88]. Similarly, Stirling et al.'s study out of the UK reported a 94 % rate of dialysis-free survival for patient who had either a complete or partial remission compared to 53 % for those who did not achieve remission ("non-responders") at 5 years [89]. It is important to note that in Troyanov et al.'s study looking at a predominately white population with FSGS, patients who achieved a partial remission had worse renal survival compared to those who had complete remission, but still had markedly better outcomes than non-responders [87].

As mentioned above, the histological sub-type of FSGS can provide some prognostic value. In general, it is believed the tip variant has the best outcome with treatment while the collapsing variant is associated with the worst outcome [94]. The perihilar type is most often caused by an adaptive change which tends to have lower amounts of proteinuria [2, 95]. Once the disease reaches nephrotic range proteinuria, it is often associated with more advanced fibrosis and sclerosis and thus lower chance of achieving remission [95]. Regardless of histological type, patients who failed to achieve any type of remission overall have worse outcomes [95, 96].

It has been reported that individuals of black race have a worse prognosis [1, 2] but this has not been the outcome in all studies [87, 88, 97]. The reason for the seemingly worse outcomes among black individuals may be related to the pathologic subtypes. There is a higher incidence of the collapsing variant and lower incidence

of the tip variant compared to white patients and thus black patients have a lower probability of achieving remission [95]. When comparing outcomes of patients with collapsing FSGS, there was reportedly no statistical difference in risk of progression to ESRD based on race [98].

The amount of sclerosis and fibrosis at time of biopsy, which often indicates more advanced disease, has been shown to be associated with worse outcomes [59]. It is important to note that studies showing worse outcomes among the collapsing variant also noted more glomerular sclerosis and interstitial fibrosis at time of biopsy [95, 98]. Renal insufficiency at time of biopsy has also been reported to be associated with lower rate of remission and worse outcomes [1, 59, 87, 98, 99]. Schwartz et al.'s multivariate analysis found a serum creatinine > 1.3 mg/dl at presentation was associated with a 10.7 relative risk for progression to ESRD in all patients. Among nephrotic patients, there was 12.7 relative risk increase for progression to ESRD when there creatinine was >1.3 mg/dl, which was more strongly correlated with ESRD than interstitial fibrosis ≥20 % (RR 6.57) [99].

7.6.4 Treatment

Treatment can be challenging in the clinical setting since the ultimate cause of FSGS may be difficult to determine and the best treatment option should ideally be tailored to the cause. Figure 7.2 outlines an algorithm to help determine appropriate treatment [2, 4].

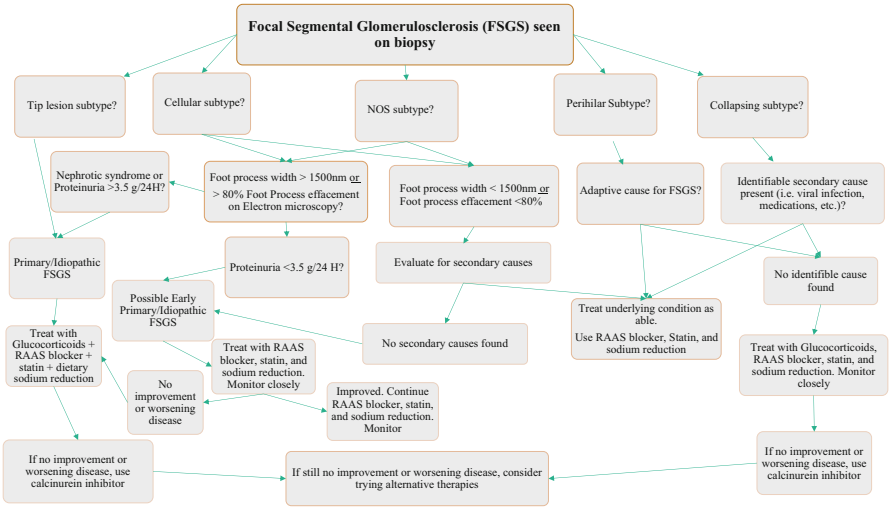

Fig. 7.2 Algorithm for diagnosis and treatment of FSGS

7.6.4.1 Conservative Management

At present, there are no large, randomized trial data on the use of renin-angiotensin-aldosterone system (RAAS) blockade in patients with FSGS. The recommendation to use RAAS blockade in FSGS is largely based on data from other proteinuric kidney diseases. It is important to note that these studies on average had patients with sub-nephrotic range proteinuria and very few patients with a diagnosis of FSGS. Furthermore, many of these trials excluded patients with massive (>10 g/24 h) proteinuria and/or those being treated with immunosuppression [60, 100]. In a prospective cohort study, angiotensin converting enzyme inhibitor (ACEi) or angiotensin II receptor blocker (ARB) use seemed to provide a renal survival benefit but this did not meet statistical significance in multivariate analysis [87]. However, given the paucity of high quality data in FSGS and the relatively strong data for their use in other proteinuric kidney disease, RAAS blockade is generally recommended in patients who do not have contraindications for their use.

Similarly, blood pressure goals for patients with FSGS have not been defined by randomized trial data. The recommendations on treatment of FSGS is largely based on data from other proteinuric kidney diseases and observational data suggesting those with higher blood pressure tended to have worse outcomes [91]. However, on multivariate analysis of prospective data, lower MAPs were not associated with renal survival [87]. Thus, BP goals are extrapolated from KDIGO recommendations to treat blood pressure to goal of <130/80 in all chronic kidney disease patients and target a goal of <125/75 in those with more than a gram of proteinuria [101]. KDIGO's guidelines also recommend lifestyle modifications including sodium reduction, normalization of weight, and smoking cessation [101].

It is felt that patients with the secondary types of FSGS derive the most benefit from conservative management strategies since secondary FSGS is often the result of maladaptive glomerular hypertension and is not likely to respond to immunosuppression therapy.

7.6.4.2 Immunosuppression

Corticosteroids Oral corticosteroid therapy has become the first-line therapy for most individuals with idiopathic FSGS and generally given at a dose of 1 mg/kg/day of prednisone for 2–3 months with a slow taper over another 4 months [102]. However, the majority of data supporting the use of oral corticosteroids for FSGS comes from nonrandomized, retrospective series [60]. Many of these studies differed in dosing of steroids, duration of treatment, and the definition of complete and partial remission. The only randomized, prospective (open label) trial of steroids in FSGS was conducted by Nayagam et al. In this trial, 16 adults were randomized to prednisolone 1 mg/kg/day for 3–6 months followed by a taper and 17 participants were randomized to mycophenolate mofetil (MMF) and low dose prednisone. Complete or partial remission was observed in 69 % of patients in the prednisolone group and 71 % in the MMF + prednisolone group [103]. Oral steroids have not been

shown to be superior to RAAS inhibition in all trials. A single center retrospective cohort by Stiles and colleagues looked at 22 patients with FSGS that had proteinuria greater than or equal to 3 g/24 h who were treated either with steroids for 4 months at a dose of approximately 1 mg/kg/day and ACEi versus conservative management with ACEi and statin therapy alone. Neither group of patients achieved complete remission and the rate of partial remission and progression to ESRD was similar between the two groups [104].

Alternatives to Steroids The use of high dose steroids is often problematic in those with diabetes, osteoporosis or prolonged previous use of corticosteroids. Unfortunately, data using other agents such as calcineurin inhibitors (CNIs) or MMF as the initial agent to treat FSGS are scarce. There is only one trial of tacrolimus (a calcineurin inhibitor) as initial therapy for FSGS. In this trial, 6 adults were treated with tacrolimus (to achieve a mean trough level of 5.5 ng/ml) for ~13 months [105]. All 6 patients achieved a partial remission (mean time to remission of 6.5 months). All participants remained on tacrolimus for the duration of the study. Thus, the optimal duration of CNI therapy for initial treatment of FSGS remains unknown. Since CNIs cause vasoconstriction, they should not be used in those with estimated glomerular filtration rate (eGFR) < 30 ml/min/1.73 m^2, in those with a rapidly rising creatinine or in individuals with moderate to severe fibrosis on renal biopsy. CNIs should be used with caution in those with moderately reduced eGFR and the serum creatinine should be monitored closely. Data on MMF or mycophenlic acid as initial treatment of FSGS are also scarce. As mentioned above, Nayagam et al., have used MMF in conjunction with prednisolone for initial therapy [103].

Relapse Unfortunately, relapse after treatment is common in FSGS [60]. In the study by Troyanov et al., over a median follow-up of 65 months, 55 of 281 subjects had a complete remission and 117 had a partial remission [87]. The cumulative relapse rate was 47 %. There are no controlled trials examining the optimal treatment of FSGS after relapse. Many clinicians will use a repeat course of steroids if the initial course was well tolerated. Options for individuals who cannot tolerate a repeat course of steroids include CNIs (if no contraindications exist to CNI use) and MMF.

Steroid-Dependent FSGS Individuals are deemed steroid-dependent if they relapse duing tapering of oral steroids or shortly after treatment. Data on the optimal approach to treating steroid-dependent FSGS are limited. Calcineurin inhibitors can be used if patients have preserved kidney function and little fibrosis on renal biopsy. Published data on the use of CNIs for steroid-dependent nephrotic syndrome often include patients with minimal change disease. Nonetheless, the use of CNIs in steroid-dependent nephrotic syndrome has been shown to induce remission rates of 70–80 % at 12 months [60]. Other options include the use of alkylating agents such as cyclophosphamide. Ponticelli et al. assigned 77 patients with steroid-dependent nephrotic syndrome (some of whom had FSGS) to oral cyclophosphamide 2.5 mg/kg/day for 8 weeks or cyclosporine 5 mg/kg/day for adults and 6 mg/kg/day for children for 9 months, tapered by 25 % per month until discontinuation by month 12. Rates of

remission were similar between the two groups but at 2 years significantly more patients in the cyclophosphamide group had not had any relapse of nephrotic syndrome [106]. Rituximab has also been used for steroid-dependent nephrotic syndrome but its use in the treatment of FSGS needs additional investigation.

Steroid-Resistant FSGS Individuals with steroid-resistant FSGS have persistent nephrotic syndrome despite treatment with oral prednisone (1 mg/kg/day or 2 mg/kg every other day) for 4 months. Steroid resistance is thought to occur in 40–60 % of individuals with FSGS and is associated with a significantly increased risk of progression to ESRD. As in the case of steroid-dependent FSGS, there are few trials that focus specifically on the treatment of steroid-resistant FSGS. Cattran et al. randomized 49 adults with steroid-resistant FSGS and eGFR > 42 ml/min/1.73 m^2 to cyclosporine + low dose prednisone versus placebo + low dose prednisone for 26 weeks [107]. At 26 weeks, 75 % of the cyclosporine group versus 22 % of the placebo group had a partial or complete remission of proteinuria. Among patients that had a remission, relapse occurred in 43 % of the cyclosporine group and 40 % of the placebo group by week 52.

MMF has been used to treat steroid-resistant FSGS in the National Institutes of Health (NIH) Focal Segmental Glomerulosclerosis Clinical Trial. In this study, 138 children and young adults with steroid-resistant FSGS were randomized to prednisone (up to 15 mg/day) + cyclosporine or MMF/dexamethasone pulses for 12 months [108]. No significant difference in rates of cumulative remission was seen between the two groups. This study, however, may not be widely applicable to the treatment of FSGS as the definition of steroid-resistance was the presence of proteinuria after only 4 weeks of steroid treatment, the trial included a large number of children, several patients in each group had sub-nephrotic proteinuria, and dexamethasone is not widely used in the treatment of nephrotic syndrome.

Alkylating agents have been used in one small trial of steroid-resistant or steroid-dependent patients with nephrotic syndrome [109] but the remainder of data on the use of cyclophosphamide for steroid-resistant FSGS comes from observational studies. Rituximab, a monoclonal antibody against CD20, has also been used for steroid-resistant FSGS in a few small trials. Gulati et al. treated 33 patients with steroid-resistant or steroid-dependent nephrotic syndrome with rituximab [110]. Six months after treatment, 49 % of patients had a partial or complete response and 51 % has no response. After a mean of 21 months of follow up, 15 patients had a sustained complete or partial remission.

Adrenocorticotropic hormone (ACTH) has also been used for the treatment of FSGS. A case series by Hogan et al. reports the use of ACTH gel in 24 adults, most of whom had steroid-resistant or steroid-dependent FSGS [111]. There were 5 partial and 2 complete remissions with 2 patients experiencing relapse during the 66 month follow up period. Drawbacks to ACTH use include the high cost and the development of side effects seen with high-dose steroids.

Recurrence After Transplantation The risk of FSGS recurrence after transplantation is estimated to be between 30 % to 50 % [112]. Recurrence of massive proteinuria soon

after transplantation is thought to be due to a permeability factor and is often treated with plasma exchange. Cyclophosphamide has been tried with variable success to treat recurrence in the allograft and rituximab has also been used. Abatacept, an antibody against the T cell costimulatory molecule CD80 (B7-1) has also been used in a small trial which included patients with FSGS recurrence after transplantation [113]. CD80 upregulation in the podocyte leads to downregulation of β1 integrin. Thus abatacept is thought to stabilize the podocyte by increasing β1 integrin expression.

Current Trials Current clinical trials investigating treatments for FSGS include comparing sparsentan (a dual endothelin receptor antagonist and ARB) with irbesartan (ARB), using adalimumab (a tumor necrosis factor α (TNFα) inhibitor), using an antibody against transforming growth factor β (TGFβ), and studies using rituximab. Details of current clinical trials in FSGS can be found at the ClinicalTrials.gov website (https://www.clinicaltrials.gov/ct2/results?term=fsgs&Search=Search).

7.7 Summary

FSGS is due to multiple causative factors that damage podocytes, ultimately leading to podocyte death and scarring of the glomerulus. There are few uniformly effective treatments for FSGS but as understanding of this heterogenous group of diseases increases, tailored, more effective therapeutics are likely to be developed.

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