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ULTRASTRUCTURAL STUDIES OF THE BLOOD-URINE BARRIER IN PROTEINURIC STATES

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ABSTRACT

The kidneys filter enormous amounts of blood every day, removing excess fluid and waste products while retaining cells and large proteins in the circulation. The podocytes with their foot processes and slit diaphragms (SD) are important components of the renal filter and the barrier function. Proteinuria is a leading sign of kidney disease, irrespective of the cause, and can in itself be harmful, accelerating the disease towards renal failure and the need for dialysis or transplantation.
In this project, we used semiquantitative immunoelectron microscopy (iEM) to describe the distribution of novel podocyte proteins in diseases with foot process effacement (FPE), the most common ultrastructural finding in proteinuria.

Study I: Expression of nephrin in minimal change nephrotic syndrome (MCNS). Under the light microscope, nephrin immunofluorescence (IFL) turned from a linear pattern along the glomerular capillary loops, into a course granular one. iEM showed a decreased nephrin labeling in MCNS, even in ultrastructurally normal podocytes. This implies a role for nephrin and the SD in the pathogenesis of MCNS.
Study II: Dendrin is a novel intracellular protein in the SD region. We analyzed its expression in MCNS compared to ZO-1, a protein known to be stable in proteinuria. In areas with FPE, dendrin was redistributed from the SD to the podocyte cytoplasm, but in contrast to nephrin, there was no overall decrease. Thus, when compared to nephrin, dendrin seems less involved in the pathogenesis of FPE.
Study III: To enable studies on novel podocyte proteins before and around the onset of proteinuria, an experimental model was used; puromycin aminoneucleoside nephrosis in rat (PAN). Nephrin, dendrin, plekhh2 and α-actinin-4 were studied. Thorough ultrastuctural analyses at different time-points in PAN were performed. α-actinin-4, a cytoskeleton cross-linker, did not change. Nephrin started to decrease on day two after induction, i.e. before the appearance of proteinuria. Dendrin and plekhh2 decreased on day four when the animals had massive proteinuria. We concluded that a disturbed expression of nephrin in PAN rats, similar to our findings in MCNS, seems to be related to the appearance of proteinuria. Changes in plekhh2 and dendrin on the other hand, could be secondary to FPE and loss of SDs.
Study IV: Pdlim2 is a cytoskeleton-associated protein, not previously shown in the kidney. Here, its association with the actin cross-linker α-actinin-4 in the podocytes is demonstrated by several techniques. Under the electron microscope, it is found centrally in the foot processes, in association with the actin cytoskeleton. In patients with MCNS and membranous nephropathy (MN), the expression was significantly reduced, while it was preserved in FSGS patients. This implies that pdlim2 may have a specific role in the pathogenesis of MN and MCNS.

In conclusion, we have developed a semiquantitative immuno-EM technique to study the expression of novel glomerular proteins in proteinuric rats, in normal human kidney, and in renal biopsies from patients with acquired renal diseases. Our studies have brought new information about the subcellular localization of these proteins. Furthermore, the expression patterns differ between diseases, indicating different roles in the pathogenesis of proteinuria.

LIST OF PUBLICATIONS

I. Wernerson A, DUNÉR F, Pettersson E, Mengarelli S, Berg U, Ruotsalainen V, Tryggvason K, Hultenby K, Söderberg M: Altered ultrastructural distribution of nephrin in minimal change nephrotic syndrome. Nephrol Dial Transplant 2003 18(1); 70-76

II. DUNÉR F, Patrakka J, Xiao Z, Larsson J, Vlamis-Gardikas A, Pettersson E, Tryggvason K, Hultenby K, Wernerson A: Dendrin expression in glomerulogenesis and in human minimal change nephrotic syndrome. Nephrol Dial Transplant 2008;23:2504-2511.

III. DUNÉR F, Lindström K, Hultenby K, Hulkko J, Patrakka J, Tryggvason K, Haraldsson B, Wernerson A, Pettersson E: Permeability, ultrastructural changes, and distribution of novel proteins in the glomerular barrier in early puromycin aminonucleoside nephrosis. Nephron Exp Nephrol 2010, *in press.*

IV. Sistani L, DUNÉR F, Udumala S, Xiao Z, Hultenby K, Uhlén M, Tryggvason K, Wernerson A, Patrakka J: Novel podocyte protein pdlim2 is differently expressed in proteinuric diseases and stabilizes stress fibers through interaction with alpha-actinin-4. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
BSA	Bovine serum albumin
CD2AP	CD2-associated protein
EM	Electron microscopy
ESL	Endothelial surface layer
FPE	Foot process effacement
FSGS	Focal segmental glomerulosclerosis
GA	Glutar aldehyde
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
HRP	Horse-radish peroxidase
IFL	Immunofluorescence
LPS	Lipopolysaccharide
MCNS	Minimal change nephrotic syndrome
MN	Membranous nephropathy
PAN	Puromycin aminonucleoside nephrosis
PF	Paraform aldehyde
SD	Slit diaphragm
TEM	Transmission electron microscopy
TRPC6	Transient receptor potential channel 6
VEGF	Vascular endothelial growth factor
ZO-1	Zonula occludens-1

1 BACKGROUND

1.1 SHORT HISTORICAL PERSPECTIVE

The British physician Richard Bright (1789-1858) is sometimes called "The Father of Nephrology". The term "Bright's disease" was formerly used to describe the symptoms of what is now more commonly called the nephritic syndrome; hematuria, casts, proteinuria, and fluid retention. After Bright's publication "Reports of Medical Cases" in 1827, the association of proteinuria and hematuria with kidney disease was established. Bright did not perform any histopathological studies and hence no further classification of the renal diseases was made.

In 1915, Volhard and Fahr classified renal diseases into nephrosis, nephritis and atherosclerotic kidney disease, with nephritis being further divided into diffuse or focal nephritis. However, the diagnoses were still presumptive and symptom-based. Arhur Ellis further classified "Bright's disease" in a Lancet publication in 1942 (1), dividing glomerulonephritides into two categories: Type I and Type II. Type I has an acute onset, usually following streptococcal infections, and a good prognosis. Type II more resembles nephrotic syndrome, with proteinuria, edema, hyperlipidemia and hypoalbuminemia. There is no hypertension or hematuria, but although renal function is described as normal, most cases eventually develop renal failure.

Until this point, histopathological studies of the kidneys were limited to post-mortem tissue, thus mostly showing late-stage renal disease (2). This changed in the 1950s when percutaneous renal biopsies became routine, allowing studies of renal diseases in earlier or milder stages.

1.2 THE NORMAL KIDNEY: BASIC STRUCTURE AND FUNCTION

The kidneys are bean-shaped organs, sized around 12 x 6 x 3 cm and located retroperitoneally approximately opposite lumbar vertebrae I-III, with the left kidney slightly above the right one (3). Their exact position varies with respiration. The functional unit of the kidney is the nephron, of which there are some 1-1.3 million in each kidney (4). A nephron is like a "miniature kidney" (Figure 1); it consists of a renal corpuscle (glomerulus), the Bowman's capsule, and its connected tubular system, the latter of which eventually drains into a collecting duct, shared by a number of other nephrons. The blood flow through the two kidneys is approximately one-fifth of the cardiac output. Basically, the blood is filtered in the glomerulus to form primary urine, approximately 135-180 L/24h (4) which is subsequently modified on its way through the tubular system, and reaches its final composition, the secondary urine, approximately 1-1,5 L/24 h, after having passed the collecting ducts.

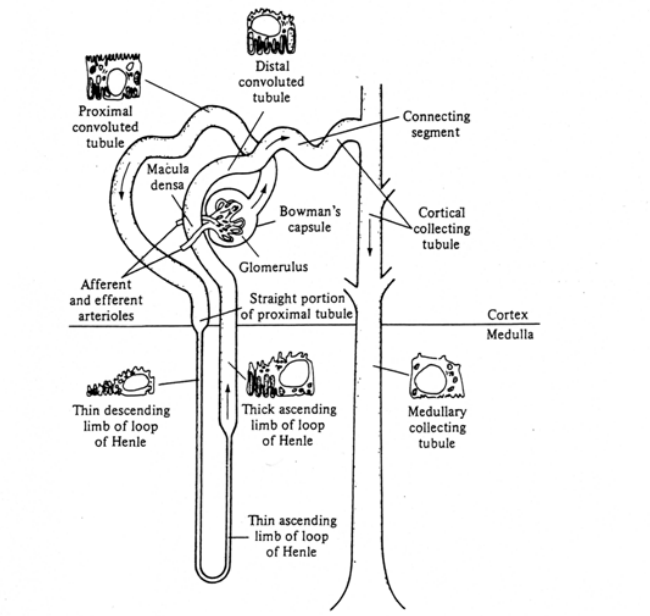

Figure 1. The nephron. One glomerulus and its tubular system. (Rose, BD: Clinical physiology of acid-base and electrolyte disorders. 4 ed. New York: McGraw-Hill; 1994).

The mammalian kidneys perform a number of major tasks. The first is to remove waste products such as nitrogenous compounds from the protein metabolism and other metabolites. The kidneys also maintain the fluid and electrolyte homeostasis and participate in blood pressure regulation via the production and secretion of hormones that regulate renal and systemic hemodynamics, such as renin, angiotensin II, and

bradykinin. The bone marrow-stimulating hormone erythropoietin is synthesized in the kidneys and vitamin D is activated through hydroxylation. Finally, the kidneys are important in regulating the acid-base balance.

Many of the tasks listed above involve glomerular filtration of the blood into primary urine, which ideally should contain all that the body wants to get rid of but nothing else.

1.2.1 Structure of the glomerular filter

On its way from blood to urine in the glomerular capillaries, the filtrate needs to pass three layers (Figure 2); the endothelium with its glycocalyx (or ESL, endothelial surface layer), the glomerular basement membrane (GBM), and finally the glomerular visceral epithelial cells, the podocytes, with their interposed slit diaphragms (SD). Before 1998, it was widely believed that the GBM was the major part of the barrier. However, the cloning of nephrin, an important molecule in the SD, and subsequent functional studies by Karl Tryggvason and co-workers (5), shifted the focus to the podocytes, and the SDs in particular. Today, many researchers advocate a more integrated view of the components of the kidney filter (6).

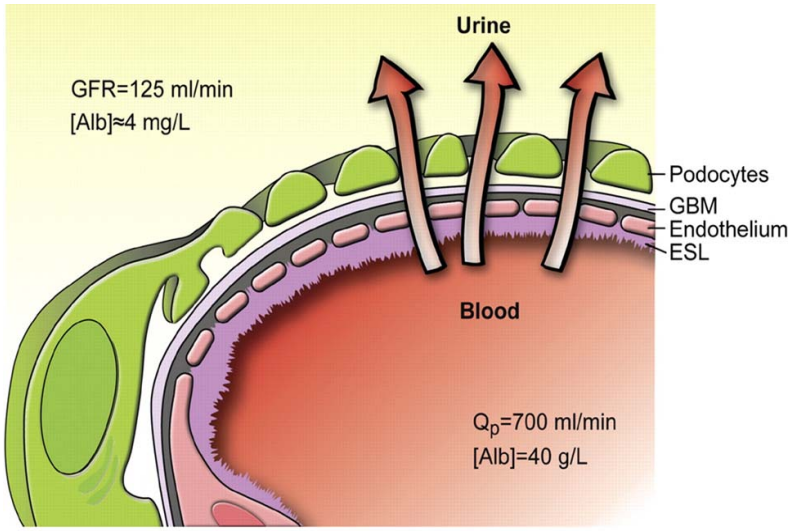

Figure 2. Schematic drawing of the glomerular barrier.
ESL = endothelial surface layer.
(Haraldsson B et al., Physiol Rev. 2008)

1.2.1.1 The glomerular endothelial cells

The glomerular endothelial cells differ from most other endothelial cells in that they have numerous fenestrae, covering some 50% of the total area (7). The pores have a diameter of ~60-80 nm (8), considerably larger than the size of an albumin molecule, but the endothelium can restrict the passage of macromolecules due to a surface cell coat of highly negatively charged glycosaminoglycans and proteoglycans. The protein orosomucoid, which is synthesized mainly in the liver but also in endothelial cells (9), seems to be of great importance for normal capillary permeability (10-11). Estimates of the thickness of the endothelial surface layer vary from ~60-100 nm (12-13) up to 0,5-1 μm (14), depending on the techniques used. Severe proteinuria is one of the symptoms seen in pregnant women with preeclampsia. The morphological hallmark of preeclampsia is glomerular endotheliosis with swelling and vacuolization of the endothelial cells (podocytes and GBM are, at least initially, intact), suggesting that proteinuria in this case is due to an endothelial damage (15). In addition, increased levels of circulating "decoy" receptors for vascular endothelial growth factor A (VEGF-A) have been found patients with preeclampsia (16-18). VEGF, which is produced and secreted by podocytes, is essential for formation and maintenance of normal endothelium (19). VEGF-null mice also demonstrate major podocyte defects, suggesting that endothelial cells signal back to the podocytes or that there is autocrine VEGF signaling from the podocytes (16, 18).

1.2.1.2 The glomerular basement membrane

The GBM is a lamellar structure with a collagen IV backbone, linked to proteoglycans and laminin (6, 20). Mutations in the collagen chains may result in Alport's syndrome (21) or the more benign "thin membrane disease" (22-23). However, the collagen network does not seem crucial for size or charge selectivity since for example patients with Alport's syndrome only have mild proteinuria. Laminin 11 (α5, β2, γ1 chains),

specifically connecting the podocyte to the GBM, may be more important; laminin β2 knockout mice display severe nephrotic syndrome even without podocyte morphology changes (24). Pierson's syndrome, a lethal form of human congenital nephrotic syndrome, has also been shown to be caused by mutated laminin β2 chain (25). The GBM could be viewed as a pre-filter of larger plasma proteins (26).

1.2.1.3 The podocytes

In the 1970s and 1980s, charge selectivity of the glomerulus was much in focus, based on the work of Barry Brenner and associates (27). This changed in 1998 with the discovery of nephrin, the protein which is deficient in the congenital nephrotic syndrome of the Finnish type (5) and the key component of the slit diaphragm, connecting adjacent podocytes.

The epithelial cell, or podocyte, wraps around the glomerular capillaries with the cell body floating in the Bowman's space like an octopus. The cell body sends out primary processes that in turn branch into foot processes which adhere to the capillaries. The foot processes of adjacent podocytes interdigitate, separated by the filtration slits that are bridged by the SDs (Figure 3). Unlike the cell bodies, the processes contain few organelles. The podocyte is terminally differentiated, and so its

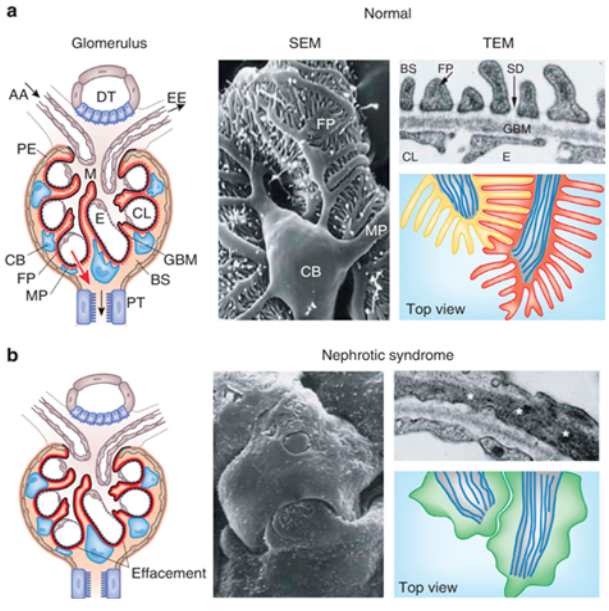

Figure 3. Podocyte structure in health (a) and disease (b): Schematic drawing (right), scanning electron micrograph of a glomerular capillary (middle), transmission micrograph of the filtration barrier (top left) and a schematic drawing of the foot processes seen from above (bottom left). **CB** = cell body, **CL** = capillary lumen, **E** =endothelium, **FP** = foot process. (Mundel P et al., Kidney Int. 2010 Apr;77(7):571-80).

potential for repair is very limited (28). The foot process structure is polarized into an "apical" and a "basal" domain. The apical cell surface is highly negatively charged, due to the expression of glycoproteins such as podocalyxin (29); this is thought to repel adjacent foot processes from each other, thereby keeping the filtration slits open (30-31). The basal domain, the "sole" of the foot process, attaches to the underlying GBM through β1α3 integrin dimers (to laminin 11, see 1.2.1.2 above) and through dystroglycan (to agrin, a heparane sulphate proteoglycan) (32). This basal domain is also important in ensuring a normal filtration barrier, as defects in integrin-mediated podocyte-GBM adhesion have been shown to result in proteinuria (25, 33).

The specialized structure of the podocyte depends on its cytoskeleton. In the cell body and primary processes, microtubules and intermediate filaments dominate. Foot processes have a delicate contractile apparatus composed of F-actin microfilaments. The primary function of the cytoskeleton is the coupling of the SD complex with the podocyte-GBM contacts (34). Microfilaments are normally bundled by cross-linking molecules such as α-actinin-4, which is highly expressed in podocytes. Podocyte damage changes the microfilaments from coordinated fibers into a dense network, which changes the cell shape resulting in fusion, or effacement, of the foot processes and narrowing of the filtration slits (35). Mutations in *ACTN4*, the gene encoding α-actinin-4, cause an autosomal dominant form of focal segmental glomerulosclerosis (FSGS) (36) which leads to nephrotic syndrome and renal failure during young adulthood. Mutated α-actinin-4 in these patients seems to have an increased binding capacity for actin, leading to abnormal actin assembly in podocytes (37). Studies performed in α-actinin-4 knockout mice have shown that α-actinin-4 has a role in podocyte adhesion to the GBM (38).

Pdlim2, also known as *mystique*, is a cytosolic ~40 kDa protein containing PDZ and LIM domains, suggesting actin binding and protein-protein

interactions. Its precise function is unknown, but it is thought to be a bundling protein of the cytoskeleton, as it associates with the α-actinin group of proteins (39-40).

Plekhh2 is a newly identified 120 kDa protein in the glomerulus. It is intracellular, observed along the glomerular capillary loops, possibly connecting the SD to the podocyte cytoskeleton (41). Zebra fish plekhh2 knock-downs have shown proteinuria and changes in the capillary walls with thickening of the GBM and disorganization of the foot processes (Parikka *et al*., submitted).

Another interesting protein in the podocyte is the transient receptor potential channel 6 (TRPC6), a calcium-selective ion channel. It is said to be localized close to the SD, though the electron micrographs presented in support of this view are not very convincing (42). Mutations in this gene give rise to another form of autosomal dominant form of FSGS (see 2.1.2 below).

1.2.1.4 The filtration slit and the SD

The filtration slit is 30-40 nm wide and is bridged by the SD. The SD was first described by Rodewald and Karnovsky (43) as a zipper-like structure with a central bar with rod-like units connected to it; this has been confirmed by recent studies with modern techniques. Electron tomographic imaging of the structure (Figure 4) reveals convoluted strands extending from the podocyte cell membranes to cross the midline of the slit, forming a

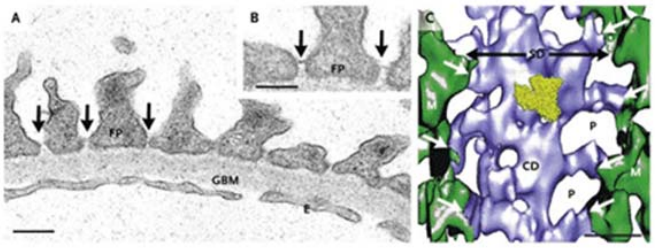

Figure 4. A and B. TEM of the filtration barrier with filtration slits (arrows) between the foot processes (FP). **4C. Electron tomogram (reconstruction) of the mouse SD, *en face***. Strands (arrows) from the podocyte cell membrane (M), meeting the central density (CD) and forming lateral pores (P). For comparison with the the pore size, an albumin molecule of the same magnification has been superimposed (yellow). Size bar = 10 nm.

(Tryggvason K et al., N Engl J Med 2006, modified from Wartiovaara J et al., J Clin Invest 2004).

central density and lateral pores with about the same diameter as an albumin molecule. Immunoelectron microscopy has been used to show that the distal parts of nephrin are in the central region of the SD, but the exact positions of the other SD-associated proteins listed below are not known (44). Functionally, the SD resembles both a modified adherens *and* a tight junction. Most of its properties suggest a tight junction, including a barrier function of selective permeability and signaling functions (45). As mentioned, the molecular composition of the SD was largely obscure until the discovery of nephrin (46-48). Since then, several other proteins of importance have been characterized.

1.2.1.5 Proteins in the slit region (Figure 5):

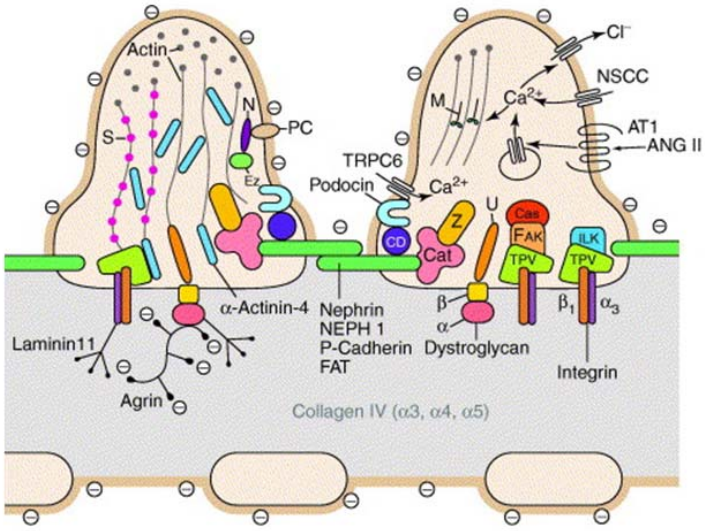

Figure 5. Two podocytes bridged by the SD, the GBM and the endothelium. Some of the molecules in our studies, e.g. dendrin, plekhh2 or pdlim2, are not included in this picture, which was published in 2005. Abbreviations (selection): **Cat** = catenins, **CD** = CD2AP, **PC** = podocalyxin, **Z** = ZO-1. (Kriz W.: Trends Mol Med. 2005).

Nephrin is a 180 kDa transmembrane protein of the immunoglobulin superfamily with a short intracellular c-terminal, a transmembrane, and a long extracellular domain, extending about 35 nm into the filtration slit (49). Nephrin molecules from adjacent foot processes bind to each other, forming the filtering structure (50). The intracellular portion is rich in tyrosine residues, which in case of phosphorylation send antiapoptotic signals to the podocyte (51). Nephrin tyrosine phosphorylation seems to control podocyte cell morphology through

interaction with members of the Nck protein family, which mediate actin polymerization (52-53). Nephrin is essential for the formation of the SD, since inactivation of its gene *NPHS1* leads to absence of a SD with severe nephrotic syndrome and neonatal death as a result (54).

Neph 1, 2, and 3 (filtrin) have structural similarities with nephrin as they are also transmembrane IgG-like molecules (55-56). Curiously, Neph 1 and 2 can form heterodimers with nephrin, but not with each other (57). Mice lacking a functioning *NEPH1* gene develop nephrotic syndrome similar to that seen in nephrin mutations (55), but the functional roles of Neph 2 and 3 are unknown.

Podocin is located exclusively in the SD region. The corresponding gene, *NPHS2*, was found to be mutated in autosomal recessive steroid-resistant congenital nephrotic syndrome (58). Podocin has a hairpin-like structure, with the ends situated intracellularly and the middle part in the plasma membrane. It interacts with nephrin, Neph1, and CD2AP (56, 59)

CD2AP was first described as part of a T-cell receptor, but surprisingly knockout experiments revealed that deficient mice died soon after birth due to nephrotic syndrome (60). CD2AP has been shown to interact with nephrin and podocin (61), and probably links them in some way to the cytoskeleton (62). No distinct human disease has been associated with CD2AP deficiency, but there are reports on human FSGS patients who carry only one allele, possibly rendering them vulnerable to glomerular injury (63). This vulnerability is supported by experimental studies (64).

ZO-1, JAM-A, cingulin, and CASK, all proteins identified in tight junctions, have also recently been associated with the SD. The role of ZO-1 is largely unknown, though it is believed to link the SD proteins and the other tight junction proteins to the cytoskeleton, and perhaps to be involved in the organization of transmembrane signaling (34, 65).

Dendrin, an 81-kDa cytosolic protein, was initially identified in the central nervous system (66-67) but surprisingly has also been detected in kidney tissue (41). Within the kidney, it is seen exclusively in the podocytes, interacting with nephrin and CD2AP (68). In neurons, it interacts with the α-actinin protein family (69), possibly modulating the cytoskeleton and thereby the shape of the cells.

1.2.2 Functional properties of the filter

The ability of a solute to pass the glomerular barrier depends on its size, charge, and shape. A more complete review on this subject is given elsewhere (6).

Size selectivity is an estimation based on theoretical mathematical calculations following perfusion experiments using tracers. The tracers molecules used include proteins such as labelled albumin, dextrans, bikunin and ficolls. However, due to tubular reabsorption, proteins tend to underestimate permeability, which of course will lower the concentration in urine. According to a recent review, the substance closest to the ideal solute is Ficoll, a neutral, heavily cross-linked sucrose-epichlorohydrin copolymer (6). Ficolls do not bind to plasma proteins, and are not reabsorbed, secreted or degraded in the kidneys. The transport of Ficolls across the glomerular barrier and other biological membranes is quite similar to that of uncharged spherical proteins (6). According to another recent review, however, the structure of Ficoll is more flexible than previously believed, particularly if charged (30).

The charge of a molecule can affect its ability to pass the glomerular barrier, since the permeability, or sieving coefficient, is much lower for anionic proteins than for neutral proteins. This effect is most prominent for molecular radii between 30 and 40 Å, where a charge similar to that of albumin reduces the permeability to 5-10% of that for a neutral molecule of similar size (6). This selectivity has previously been thought to reside primarily in the negatively charged GBM, but more recent studies do

not support this (6, 30). Agrin, a heparane sulphate proteoglycan, is the most important contributor to the anionic charge of the GBM. However, agrin-deficient mice have intact charge barriers (70), and so the charge of the glomerular cells (podocytes and endothelial cells) must be of greater importance, as for example in the abovementioned podocalyxin of the podocytes (71). However, there is no known human kidney disease caused by a defect in podocalyxin (30). Proteinuria might be caused by ischemia and reperfusion of the kidneys, which is clinically relevant for example following renal transplantation (72). Shorter ischemia (15-20 min) in rats has been shown to decrease glomerular charge selectivity, with no apparent damage to the podocytes or GBM, suggesting that there is an effect on the endothelial cell surface layer (73). Otherwise, there is very little data on charge selectivity in human proteinuric disease. Given the balance of available evidence, it is hard to say that either charge or size is more important than the other.

The shape of molecules also affects glomerular transport properties. For example dextrans, which are elongated, coiled molecules sometimes used as tracers, tend to overestimate permeability due to their ability to "squeeze" through the filter (74). Markedly elongated proteins, such as bikunin, have higher permeability than spherical proteins of similar size (6).

The paradigm of the glomerular barrier has lately been challenged by the albumin retrieval hypothesis from a group in Australia (75). According to their investigations, the glomerulus is far more permeable to albumin than commonly thought, and the filtered albumin is completely reabsorbed (retrieved) in the proximal tubules and returned to the circulation. Thus, nephrotic syndrome is a tubular rather than a glomerular disease. The same group has used 2-photon microscopy to show that albumin is filtered at much higher levels (50 times more) than previously thought. However, no tubular mechanism with the capacity of reabsorbing the proposed

amounts of filtered albumin has yet been described; the "reabsorptive burden" for the proximal tubules would be ~240 g/d, which is unrealistic. The well-known prognostic importance of nephrotic-range proteinuria in human renal disease is also hard to explain, as for example 3.5g/24h would be only ~1.5% of the total leakage. Finally, recent papers have questioned the 2-photon microscopy method (76-77) as the authors were unable to repeat the results of the Australian group. Thus, it seems safe to be skeptical of the albumin retrieval hypothesis.

To conclude, normal function of the glomerular filtration barrier requires three intact layers, particularly the endothelium with its glycocalyx and the podocytes with their slit diaphragms.

1.3 PROTEINURIA

While low-molecular compounds and ions are able to pass freely through the glomerular filter, only minute amounts of proteins above the size of albumin (69 kDa, 4 nm) escape into the urine. In a healthy person, the urinary excretion of protein is less than 150 mg/24h and consists mainly of filtered plasma proteins (60%) and tubular Tamm-Horsfall proteins (40%) (78). Albumin is the main plasma protein in the urine, constituting about 20% of daily protein excretion.

1.3.1 Urinalysis

Excretion of more than 150 mg of total protein per 24h is considered pathological (79). Nephrotic syndrome is usually defined as proteinuria exceeding 3.5 g/24h (in children more than 40 mg/h per m^2) along with hypoalbuminemia and hyperlipidemia. Proteinuria can be classified as glomerular, tubular or overflow proteinuria on the basis of the types and origins of the proteins found in urine (80). The sections above have

dealt with glomerular proteinuria, when the glomerular barrier is damaged, leading to a passage of proteins the size of albumin and larger. Overflow proteinuria is caused by large-scale glomerular filtration of proteins of lower molecular weight, most commonly light immunoglobulin chains secreted by malignant plasma cells in myelomatosis. When the reabsorptive capacity of the tubules is exceeded, the proteins appear in the urine. Smaller proteins, typically ~25 kDa, such as β2-microglobulin, are freely filtered in the glomerulus and subsequently reabsorbed in the tubuli. Damage can occur to the tubuli, decreasing their resorptive capacity, as a result of various tubulo-interstitial diseases such as infections or heavy metal poisoning. The result of this is tubular proteinuria, which may also include smaller amounts of albumin due to impaired reabsorption of filtered albumin. Intense exercise can also result in a transient leakage of plasma proteins, with a duration of about 24-48 h, without any signs of renal disease (78).

A common, reliable and cost-effective method to detect proteinuria is dipstick analysis. However, since dipsticks are only affected by albumin, they cannot detect either overflow or tubular proteinuria.

1.3.2 Diagnostics of proteinuria: The renal biopsy

There is no more certain way to find out the underlying cause of proteinuria than through a histopathological examination. Thus, performing a renal biopsy remains the most important step when investigating possible renal parenchymal disease (2), including rejection in transplanted kidneys. Renal biopsy today is reliable and safe, as it is performed using ultrasound guidance. The size of the specimen is chosen as a compromise between the diagnostic possibilities (the bigger the better) and the risk of bleeding. Light microscopy, quick and easy to perform, gives an overview of the tissue. Immunofluorescence (IFL) is generally used to find immune complexes, i.e. antibodies

and/or complements in the specimen. Electron microscopy (EM) gives a higher magnification than light microscopy (see Materials and Methods) and enables subcellular studies.

1.3.3 Proteinuria as a risk factor in human kidney disease

Proteinuria is a leading sign of renal disease. Many patients with glomerulonephritis or diabetic nephropathy have a progressive course with a declining renal function ending in end-stage renal disease (ESRD), for which the only life-saving measure is renal replacement therapy (RRT); that is, dialysis or transplantation. According to the Swedish renal registry (SNR, www.medscinet.se/snr), 26% of RRT patients have glomerulonephritis and 19% have diabetic nephropathy. Both conditions are accompanied by proteinuria. It has been established for some time that proteinuria in itself is harmful to the kidneys, particularly the tubular system, irrespective of the underlying disease. A relationship between the amount of urinary protein and the rate of decline of glomerular filtration rate (GFR) has been reported in both diabetic nephropathy and non-diabetic glomerular disease including FSGS, membranous nephropathy (MN), lupus nephritis, and IgA nephropathy (80). By pharmacologically reducing proteinuria with angiotensin converting enzyme (ACE) inhibitors or/and angiotensin receptor blockers, preservation of kidney function has been shown in both diabetic and non-diabetic kidney disease (81-84). A relevant question is of course whether proteinuria is simply a marker of a more severe damage to the glomerular filter, rather than toxic to the tubular system. In any case, mapping the causes of proteinuria and finding treatment for them would be a significant improvement for these patients.

2 NEPHROTIC SYNDROME

2.1 HEREDITARY FORMS OF NEPHROTIC SYNDROME

2.1.1 Autosomal recessive disorders

Congenital nephrotic syndrome of the Finnish type is caused by mutations in the nephrin gene *NPHS1* (5). The incidence in Finland is 1/8200 births, but is also seen throughout the rest of the world. Two mutations account for ~94% of the cases in Finland, though more than 70 mutations are known worldwide (85). These babies have proteinuria in utero and are usually born prematurely. Following birth, the nephrotic syndrome soon becomes apparent. Histopathological analyses show foot process effacement and absence of a slit diaphragm (44, 86). Medical treatment is supportive, and includes albumin infusions, nutrition, treatment of infections, and so on. Bilateral nephrectomy followed by dialysis and renal transplantation is the treatment of choice as it is curative. However, in one-quarter of the grafts the proteinuria recurs, and in half of these cases, anti-nephrin antibodies have been detected (87).

Steroid-resistant nephrotic syndrome due to mutations in the podocin gene (58):

More than 30 mutations in the *NPHS2* gene have been described (88). The affected children develop a nephrotic syndrome in early childhood which is resistant to standard corticosteroid treatment. Renal biopsy shows FSGS. Renal failure follows, and transplantation is the treatment of choice. *NPHS2* mutations have also been found in sporadic FSGS cases, with later onset (49). Recent data from Antignac's group demonstrated that conditional inactivation of *NPHS2* in adult mice also leads to nephrotic syndrome and FSGS, associated with the disappearance of podocin from the slits (89).

Pierson's syndrome is a combination of ocular defects and congenital nephrotic syndrome leading to renal failure. The syndrome is very rare and the children die before two months of age. The underlying kidney defect is a mutation in the gene for the laminin β2 chain of the GBM (25). As mentioned above, knockout mice with the same genetic defect also show a similar phenotype (24).

2.1.2 Autosomal dominant disorders

Familial focal segmental glomerulosclerosis type 1 (FSGS1) is characterized by slowly increasing proteinuria in adolescence or early adulthood, progressive renal failure, and finally end-stage renal disease. It is caused by a mutation in the *ACTN4* gene, which encodes α-actinin-4, an actin cross-linker in the cytoskeleton of the podocyte. While the causative mutation in the type I disorder was described as long ago as 2000 (36), a second type, *FSGS2* was only recently shown to be caused by mutations in *TRPC6*, a gene encoding the Transient Receptor Potential Cation Channel 6 (42, 90), an ion channel recently located in the podocyte. Proteinuria occurs in young adulthood and 60% of the affected children progress to ESRD.

2.2 ACQUIRED FORMS OF NEPHROTIC SYNDROME IN ADULTS

In children with nephrotic syndrome, MCNS is seen in 65-90% of the cases in different series (79); conversely, only 15% of adults cases are attributed to MCNS. The most common cause in adults, affecting about one-third of cases (91), is membranous nephropathy. The exception to this is people of African origin, among whom FSGS dominates.

Minimal Change Nephrotic Syndrome (MCNS)

MCNS is defined by the absence of glomerular abnormality in light microscopic examination (92), though mild mesangial hypercellularity can be accepted. Electron microscopy shows widespread fusion, or effacement, of the podocyte foot processes. Despite the lack of inflammatory cells in the kidneys, the pathogenesis is thought to be autoimmune: MCNS often makes its debut after respiratory tract infections, it is associated with lymphomas and atopy, and it responds to corticosteroid treatment (92). Numerous abnormalities in humoral and cellular immunity have been reported, but no single pathogenic process has yet been identified. The presenting symptom is often fluid retention and edema, developing over a fairly short time period. Hematuria is generally not a feature of MCNS, but should rather raise the suspicion of FSGS. Historically, MCNS was associated with significant morbidity and mortality due to infections and thromboembolic complications. Today, steroids offer rapid help, and while MCNS may recur, it does not lead to progressive renal failure.

Focal segmental glomerulosclerosis (FSGS)

FSGS is a far more ominous diagnosis than MCNS. FSGS may be viewed as a set of histopathological patterns rather than a diagnostic entity. These patterns have partially different courses and prognoses. Histologically, classic FSGS is characterized by segmental glomerular scarring; the capillaries are obliterated and adhere to Bowman's capsule in the affected segments, while other segments or other glomeruli may be intact (93). Therefore, lesions can initially be missed and the patient misclassified as having MCNS.

Eventually, more and more glomeruli will succumb, along with their tubuli, leading slowly to end-stage renal disease in around 50% of patients within 8-10 years (92, 94). As mentioned above, there are several hereditary forms of FSGS with

known genetic defects. When it comes to sporadic cases, which are far more common, the pathogenesis is obscure. First-line treatment, apart from ACE-inhibitors and other anti-proteinuric measures, is corticosteroids to which up to 40% of patients respond (94). Five-year renal survival is >95% in the responders and around 57% in non-responders (95). However, secondary forms of FSGS (e.g. caused by obesity, drugs, solitary kidney, etc) must be excluded, as these patients will not benefit from steroids. A subgroup of patients with FSGS are thought to have a circulating pathogenic factor which has not yet been characterized. The disease may recur in the transplanted kidney, and plasma exchange can give temporary relief.

Membranous nephropathy (MN)

MN is the most common cause of nephrotic syndrome in adults and is rare in children. Two thirds of the cases are idiopathic, but there are also secondary forms. MN is characterized by granular, subepithelial immune deposits which most often contain IgG and complement system components (96). A very recent publication described type-M phospholipase A2 receptor (PLA2R) on the podocyte cell surface as the first autoantigen identified in idiopathic membranous nephropathy in the adult (97). In that study, 70% of patients with idiopathic MN carried circulating anti-PLA2R antibodies. MN has an experimental correspondence, the Heymann nephritis of the rat, in which this antigen is known as megalin, a large podocyte-membrane glycoprotein. However, megalin is neither expressed in human podocytes nor detected in immune deposits in patients (98). It also remains to be determined if exogenous antigen can be trapped in the subepithelial space and induce MN; for example hepatitis B virus inducing secondary MN. Complement factors C5b-9 are known as the membrane attack complex. This complex is inserted into the podocyte cell membrane and, since podocytes are resistant to lysis, endocytosed (96). C5b-9 induces cell activation,

including production of oxidants and proteases which damage the GBM and lead to proteinuria. This activation also makes the podocytes produce more GBM matrix, leading to the classic thickening of the GBM, which has given rise to the name "membranous nephropathy".

In an Italian study on 100 patients with idiopathic MN, 25% of the patients had a spontaneous complete remission, 25% continued with lessened but persistent proteinuria and stable GFR, while the remainder continued with nephrotic-range proteinuria and a progressive course. The progression was rather slow, with some 22% of cases ending in dialysis or death within eight years (99). The authors concluded that aggressive therapy such as steroids or cytotoxic agents should be reserved for patients with signs of poor renal prognosis; that is, sustained pronounced proteinuria and reduced GFR (99).

2.3 ANIMAL MODELS FOR PROTEINURIA AND NEPHROTIC SYNDROME

It is self-evident that many questions cannot be answered through the study of human renal biopsies, and so, experimental studies are needed. Here follows a short overview of some models that affect the podocytes.

Adriamycin nephrosis is a mouse model for nephrotic syndrome and progressive renal scarring, resembling FSGS. Only certain strains of mice are susceptible to adriamycin. Rats may also be used, but mice are more popular due to ease of handling, economy, and the potential for genetic manipulation (100). The pathogenesis of the podocyte toxicity is largely unknown; however, at least in mice, the alternative complement pathway seems to be of importance. After injection of adriamycin, or another anthracycline antibiotic, mice develop overt proteinuria on day 5, followed by

progressive glomerular hypertrophy and FSGS. By week 6, many glomeruli are completely sclerosed.

Lipopolysaccharide (LPS)-induced proteinuria was first described in 2004 as a reversible model for foot process effacement and proteinuria in mice (101). LPS is a part of the outer membrane of gram-negative bacteria, and intraperitoneal injection of LPS in mice results in proteinuria and FPE within 24h, returning to baseline within 72h (101). LPS also has effects on the circulation, including the risk of septic shock, so many organs besides the kidneys are involved. Thus, care should be taken when interpreting results (100).

Heymann nephritis of the rat (HN) was originally described in 1952 (102), and first believed to be the result of passive glomerular trapping of preformed immune complexes. Later, the immune complexes were shown to be formed in situ (96), closely resembling human membranous nephropathy. The *active* HN, induced with injection of kidney extracts, is seldom used today due to methodological uncertainties (100). It has been replaced by the *passive* HN (103), induced by the injection of rabbit antiserum against tubular epithelium (anti-Fx1A). Proteinuria occurs 4-7 days after injection and can be ameliorated by co-distribution of cobra venom, which inhibits the complement system.

Puromycin aminonucleoside nephrosis of the rat (PAN) has been used since the 1950s as a model for proteinuria, and in particular, minimal change disease (MCNS) (6, 103-104). Mice are generally resistant to PAN. In rats, however, puromycin is toxic to podocytes, probably due to DNA damage via the production of reactive oxygen species (105-106). Four to five days after a single intraperitoneal injection of the drug, the rats develop proteinuria. This proteinuria may resolve after around three weeks unless multiple injections are given, in which case the result is glomerular scarring and progressive renal failure, resembling FSGS. Initially, PAN was believed to affect only

the charge barrier, but more recent studies have revealed injuries also in the size selectivity (104). We chose PAN for our experiments because of its reversibility and resemblance to MCNS.

3 EXPRESSION OF PODOCYTE-ASSOCIATED MOLECULES IN NEPHROTIC SYNDROME

The results regarding podocyte or slit diaphragm (SD) associated molecules in human proteinuric states are variable. Initial studies used light microscopy and/or Western blotting. Patrakka *et al.* (107) found no significant change in the distribution of nephrin in MCNS, MN, or FSGS using IFL. Doublier *et al.*, studying various forms of nephrotic kidney diseases including MN and MCNS with the same methods, found that nephrin labeling changed from linear to granular, indicating re-distribution of the protein. The fluorescence intensity of nephrin was decreased, irrespective of underlying disease (108). Koop *et al.* found a decrease in nephrin protein in several disease categories (FSGS, lupus nephritis, MCNS, diabetic nephropathy) (109). mRNA, on the other hand, was mostly increased, which was interpreted as a compensatory mechanism. The authors suggested that this group of molecules react stereotypically in proteinuric diseases, but that the morphological changes are due to some other insult. Kim et al (110) found a granular distribution of nephrin along the GBM in MCNS, along with a diffuse labeling in the podocytes, indicating redistribution. In MN and FSGS, this granular pattern was more pronounced. Further, nephrin mRNA was significantly decreased in MN and FSGS, but not in MCNS. Ultrastructurally, there were fewer nephrin markers in effaced foot processes, although no quantification was performed, and the authors did not compare areas with and without foot process effacement.

Patrakka *et al.* also analyzed ZO-1; light microscopy revealed no difference in its expression in MCNS, FSGS, or MN, (107), a result in agreement with the earlier study by Bains *et al*. (111).

The results are also divergent regarding experimental disease. Most studies of PAN have shown a decreased expression of nephrin protein in podocytes on day 3-4 (112-116). However, results regarding mRNA, which reflects on-going protein synthesis, have been more variable. While some researchers have found a decrease in mRNA (112, 114-115), another group found an early *increase* in nephrin mRNA in early PAN, followed on day four by a decrease in protein levels (113). In a spontaneous proteinuric rat model, nephrin protein also decreased, while mRNA increased (117). The authors concluded that nephrin decrease was associated with the morphological phenomenon of foot process effacement rather than proteinuria. Proteinuria and foot process effacement are not necessarily correlated to each other, as foot process effacement can be present without proteinuria (118-119) and vice versa (24, 120-121). Xing *et al.* studied nephrin and α-actinin-4 in experimental adriamycin nephrosis (122), combined with ACE inhibitor therapy. The podocyte proteins were semiquantified using Western blotting and immunofluorescence. ACE inhibition ameliorated proteinuria, and also partly prevented the change of nephrin and α-actinin-4 IFL from linear to granular. It is notable that there are very few ultrastructural studies.

There is only one previous publication on dendrin in kidney disease (68). Asanuma *et al*. showed that dendrin is associated with nephrin close to the SD. In a mouse model of podocyte damage by injection of anti-rabbit glomerulus antiserum, they found accumulation of dendrin in podocyte nuclei which was enhanced by the pro-apoptotic cytokine TGF-β. In cultured podocytes, nuclear dendrin enhanced apoptosis.

4 AIMS OF THE STUDIES

The overall aim of our studies was to elucidate what happens when the glomerular filter leaks proteins. Is there a change in the expression of certain proteins that are known to be parts of the glomerular filter and thought to be of importance? Is there a difference between diseases? When and where do the first alterations appear? More specifically:

- Is the expression of nephrin altered in human nephrosis, particularly minimal change nephrotic syndrome? **(Study I)**
- Does the expression of dendrin differ from that of the slit proteins nephrin or ZO-1 in minimal change nephrosis? **(Study II)**
- What happens in early experimental nephrosis in the PAN model? Are there any alterations in the expression proteins associated with the SD and/or cytoskeleton? When do these changes occur? Can they be alleviated with an ACE inhibitor? **(Study III)**
- How does the cytoskeletal protein pdlim2 behave in various types of human nephrosis (MN, MCNS, FSGS)? **(Study IV)**

5 MATERIALS AND METHODS

5.1 PATIENTS

Patients undergoing renal biopsy for the diagnosis of nephrotic syndrome were considered for the studies. The routine biopsy needle is 16G (1.6 mm thick) and two biopsies are obtained and then divided to allow for standard light microscopy, immunofluorescence (IFL) and electron microscopy (EM). Tissue for immuno-EM has to be handled separately, see 5.4.4, and could only be taken if possible without compromising diagnostic safety for the patient. All cases were reviewed to confirm the diagnosis. Clinical and laboratory data were collected from the patient records. In Study IV, only patients with the perihilar ("classic") form of FSGS according to the Columbia criteria (93) were included.

5.2 EXPERIMENTAL STUDIES

The single-shot PAN model was used (III), with male Sprague-Dawley rats (B&K Scandinavia or Scanbur, Stockholm, Sweden) weighing ~250 g. Rats were kept in Macrolon 4 cages for at least one week before the experiments, with controlled light-dark cycles, humidity, and temperature. They had free access to tap water and standard rat chow. Before the experiments, each rat was kept individually in a metabolic cage for 24h to enable urine collection. Puromycin aminonucleoside (Sigma-Aldrich Co, St Louis, MO, USA) 150 mg/kg body weight was injected intraperitoneally at day zero (for details about PAN nephrosis, see page 20). Control animals received equal volumes of saline. 24 h before sacrifice, each rat was again kept in a metabolic cage.

All studies were approved by the Animal Ethics committees of the Karolinska Institutet or the University of Gothenburg.

A basic evaluation of the method was carried out with nine PAN and nine control rats, studied up to nine days. The rats were anaesthetized with pentobarbital (Pentobarbitalnatrium, 60 mg/ml, Apoteksbolaget, Sweden) 1 ml/kg and Hypnorm (Janssen, Beerse, Belgium) 0.5 ml/kg. A laparatomy was performed, and the left kidney was removed, cut in half and immediately frozen for IFL. Next, 2500E of heparin was given intramuscularly, and the thorax was opened. A cannula was placed in the left cardiac ventricle, and fixative (3% paraform aldehyde, PF, and 0.1% glutar aldehyde, GA) was infused at 75 cm H_2O until the right kidney was properly fixed. The kidney was then divided into three portions: one for light microscopy; one for routine EM; and one for immuno-EM.

The next series of experiments were aimed at combining physiological and morphological studies. To study glomerular permeability, the cooled isolated kidney method was employed. In this method, the kidney is perfused in vivo with tracer molecules, and by studying the fractional clearances and sieving coefficients of these, the permeability properties of the glomerulus can be described. Possible tubular interference is inhibited by the low temperature of the perfusate and by adding furosemide. The basic method, described in by Lindström (123), was slightly modified to enable morphological studies.

Anesthesia was induced with pentobarbital as described above, and maintained by isoflurane inhalation at concentrations of 0-1%. The rat, kept on a 37°C heating pad, had the tail artery cannulated to enable monitoring of the arterial pressure and administration of drugs. The rat was eviscerated through a midline abdominal incision. Diuresis was stimulated by i.v. furosemide 2 mg/kg, facilitating the cannulation of the

left ureter. A PF 50 catheter was put into the abdominal aorta. The right kidney was removed, and the aorta was simultaneously clamped between the two renal arteries, allowing selective perfusion of the left kidney. The vena cava was opened to secure free venous outflow, and perfusion was started immediately.

The perfusate was a cooled (8°C), modified Tyrode-albumin solution with the following composition (123): NaCl 113 mM, KCl 4.3 mM, $MgCl_2$ 0.8 mM, $NaHCO_3$ 25.5 mM, NaH_2PO_4 0.5 mM, $CaCl_2$ 2.5 mM, glucose 5.6 mM, and human serum albumin 20 g/l. The perfusate also contained furosemide 10 mg/l, sodium nitroprusside 0.27 mg/l, and ^{51}CrEDTA (Amersham Pharmacia Biotec, Buckinghamshire, UK) to measure GFR. Fluorescin isothiocyanate-labelled ficoll (Bioflor HB, Uppsala, Sweden) 2 g/l perfusate was added as tracer.

The excised right kidney was immediately divided and processed for histopathology as described in chapter 5.4. The "urine" from the perfused left kidney was collected in vials. Radioactivity from the CrEDTA was measured with a gamma counter (Cobra Auto-Gamma™ Counting Systems, Packard Instrument Company, Meridan, CT, USA). GFR was calculated with the formula $GFR = U*Q/C_p$ where U and C_p are the concentrations in urine and plasma, respectively, and Q is the urine flow. Ficoll was detected using high-pressure liquid chromatography and fractional clearances and sieving coefficients of the Ficoll molecules of various sizes were computed.

A series of experiments were carried out in which half of the PAN rats were given the ACE inhibitor enalapril in the drinking water (40 mg/l), starting from two days before PA injection.

5.3 PRIMARY ANTIBODIES

Through a large-scale glomerular genomics, proteomics, and bioinformatics project, a large number of "new" glomerular proteins have been identified by the research groups of Christer Betsholtz, Karl Tryggvason, and Mathias Uhlén. The function of these proteins was further tested in knock-down zebra-fish and knockout mice models, and those associated with signs of renal disease were selected for our studies.

Anti-nephrin: A rabbit polyclonal antibody directed against the N-terminal, extracellular portion of nephrin (48).

Anti-ZO-1: Purchased from Zymed (San Francisco, CA, USA).

Anti-dendrin: Rabbit polyclonal anti-mouse antibodies (41).

Anti-plekhh2: Rabbit polyclonal antibodies (41).

Anti-pdlim2: Rabbit polyclonal antibodies were produced as described in Study IV. Briefly, residues 87-212 for pdlim2 were expressed as recombinant protein with a hexahistidine tag that enabled purification of the antigen by chromatography. Antibodies were raised by immunizing rabbits. The specificity of the antibodies was confirmed using pdlim2-transfected HEK293 cells.

5.4 TISSUE HANDLING

5.4.1 Light microscopy

Specimens were fixed in 4% PF, dehydrated, and embedded in paraffin according to standard procedures. 1.5 µm sections were cut on a microtome. They were stained with hematoxylin-eosin, periodic-acid Schiff (PAS), Ladewig trichrome, and periodic-acid silver (PASM) methods.

5.4.2 Immunofluorescence

The purpose of immunohistochemistry is to define the location of a biochemically defined antigen by using a labeled antibody. If the structure of the antigen is changed, for example due to tissue handling, the antibody may not recognize it. Therefore, the tissue has to be handled with care. In our practice, routine renal biopsies are unfixed, and incubated with FITC-conjugated antibodies against IgG, IgA, IgM, fibrinogen, the light chains κ and λ, and the complement factors C3 and C1q. For the studies, specimens were frozen in liquid nitrogen. 5 µm cryosections, unfixed or fixed with -20°C acetone, were blocked with 5% normal serum from the species in which the antibody was made. The primary antibodies were incubated overnight at 4°C followed by a 1h-incubation with the secondary fluorescent antibody.

5.4.3 Immunoperoxidase staining

2.5 µm sections of paraffin-embedded tissue were pre-treated with tris-EDTA (Dako, Glostrup, Denmark) followed by 3% H_2O_2 in methanol. Blocking was performed with 10% milk protein for 30 min. The primary antibody, or normal IgG as negative control, was incubated overnight after which the HRP-conjugated secondary antibody (Envision™, Dako, Glostrup, Denmark) was added for 30 min at room temperature and then visualized by the DAB/H_2O_2 substrate. Nuclei were stained by hematoxylin.

5.4.4 Transmission (TEM) and immunoelectron microscopy

The resolution of the light microscope is limited by the wavelength of light. In the electron microscope, light is replaced by electrons, which have a much shorter wavelength. This increases the resolution, thereby allowing for subcellular (ultrastructural) studies. As no color information can be elicited, raw EM images are in grayscale only. EM relies on density differences in different parts of the tissue studied (e.g. mitochondria, nucleus, cell membranes), further increased in the processing of the

tissue using heavy metal compounds such as osmium, urane, and lead. This affects the way in which the electrons are scattered, or redirected, from their straight path through the tissue, and hence the contrast of the image (see below).

Normally, tissue intended for EM is fixed in buffered 2% of glutar aldehyde (GA), which heavily cross-links proteins. A second step, called post-fixation, is performed with the heavy metal compound osmium tetroxide (OsO_4); this also enhances contrast in the specimen. A prerequisite for TEM is ultrathin (40 nm) sectioning. To enable this without destroying the specimen, it has to be embedded in a hard plastic resin. Since these compounds are non-polar, water has to be eliminated from the tissue by serial alcohol dehydration. After embedding and sectioning, the specimen is mounted on a grid. The final step is the contrasting. The electron microscope accelerates an electron beam through the specimen, creating an image on a fluorescent screen on the basis of the degree to which the electrons are scattered by the passage through the specimen. Without the contrasting, which is accomplished by adding heavy metals such as lead citrate and uranyl acetate to the tissue, the electrons would pass straight through the ultra-thin section, resulting in a low-contrast image. For immuno-EM, tissues must be prepared with special methods from the start. The conventional protocol for TEM preparation includes several steps, such as GA, osmium tetroxide, dehydration, embedding in a non-polar resin, and finally polymerization of the resin at 60°C, all of which will influence the configuration of the proteins in the tissue. This protocol is unsuitable for immuno-EM since it will prevent the primary antibodies from binding to their target proteins. By changing the protocol to use 2% PF and 0,5% GA for primary fixation, omitting the osmium step, performing dehydration at a low temperature (-25°C), and embedding the tissue in a polar resin (Lowicryl K11M, Chemische Werke Lowi GmbH, Waldkreiburg, Germany), the proteins are much better preserved, thereby increasing the possibility for the antibodies to bind to

their target antigen. (124). Thus, tissue is cut into 40 nm sections and mounted on formvar nickel grids. To minimize unspecific labeling, the grids are incubated (blocked) with 10% bovine serum albumin in 0.1M phosphate buffer at pH7.4 for 2h at room temperature. Then, the primary antibody is added, and the samples are incubated overnight at 4°C. Control tissue is incubated with normal rat serum 1:500. To detect bound antibodies, protein A with conjugated colloidal gold markers is incubated for 2 h at room temperature. The grids are rinsed in 0.1M phosphate buffer with 0.1% BSA. To stabilize the binding of the primary antibody and protein A, the grids are postfixed in 2% GA in 0.1M cacodylate buffer with 0.1M sucrose for 10 min. Finally, the specimens are contrasted with 4% uranyl acetate followed by Reynold's lead citrate.

5.4.4.1 Semiquantitative immune-EM

The gold particles mentioned above are spherical, 10 nm in diameter, and very electron dense and therefore easy to see on EM micrographs. They are attached to a secondary IgG antibody or protein A, which is a staphylococcal protein with strong affinity to the Fc part of immunoglobulins. There are two reasons to use protein A instead of a secondary antibody. First, each protein A molecule binds one primary antibody, so each gold marker denotes one bound primary antibody and can be used as a basis for semi-quantification of the studied protein. The second advantage of this choice is the specificity it gives: protein A produces lower background labeling than secondary antibodies. A non-specific bound primary antibody

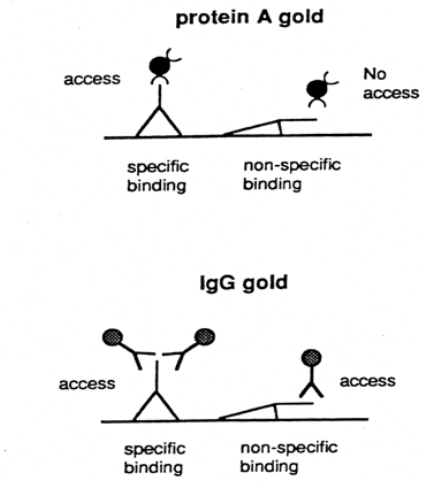

Figure 6. Non-specifically bound primary antibodies are less likely to be labeled by protein A gold than by IgG gold. (From Griffiths G.: Fine structure immunocytochemistry. Heidelberg: Springer-Verlag; 1993).

would be less likely to expose its Fc part than the specifically bound antibody that we want to detect. Protein A can only bind to the Fc part and this makes the risk of unspecific detection smaller (125) (Figure 6). A drawback, on the other hand, is that the labeling might appear scarce on the micrographs, which makes it hard to get a "quick impression" of the amount of gold particles.

The sections for immuno-EM were examined in a LEO 906 transmission microscope (LEO Electron Microscopy Ltd, Oberkochen, Germany) at 80 kV. Stereology refers to a body of mathematical methods enabling quantification of three-dimensional structures from measurements on two-dimensional sections of the structure (126). In the present investigation we used a tool to measure area which often is applied in stereology, i.e. point counting. Also important in stereology is to use a proper method for sampling. In our studies we used random sampling. Based on the literature and previous studies (126), a cumulative mean plot was made to determine the number of images needed for an appropriate sample. The images (x40 000) were taken with a digital camera and processed with iTEM software (Olympus Soft Imaging System, GmbH, Münster, Germany). When present, three glomeruli were examined from each rat/patient, and five images were taken from randomly picked spots in the periphery of each glomerulus (Figure 7). If the sections contained less than three glomeruli, 15 images were taken from those that were

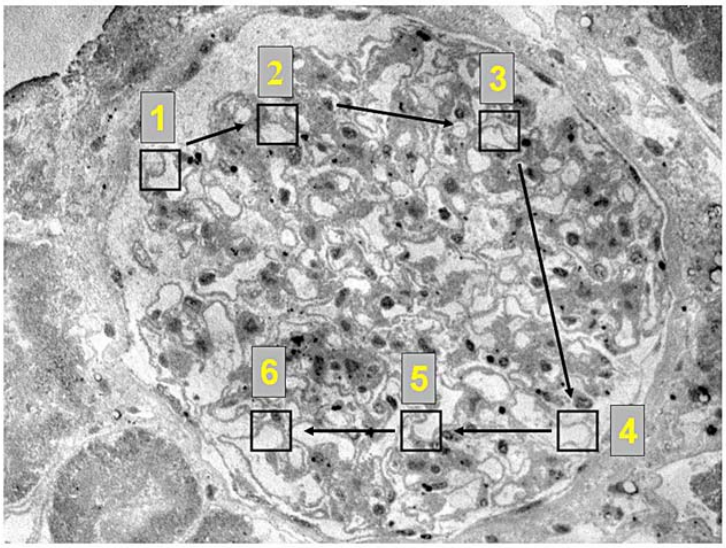

Figure 7. Sampling of glomerular capillaries for semiquantification of proteins. Five to six spots are randomly picked from each glomerulus in low magnification where no gold labels are visible.

present. In Studies I and II, areas with or without foot process effacement were separated, and 2x15=30 images were taken from each case, to ensure 15 in each group. To arbitrarily delimit the foot processes, a line was drawn "above" and parallel to the GBM , and the areas of the foot processes within this zone were measured by the point counting method (126) using a transparent lattice grid (Figure 8).

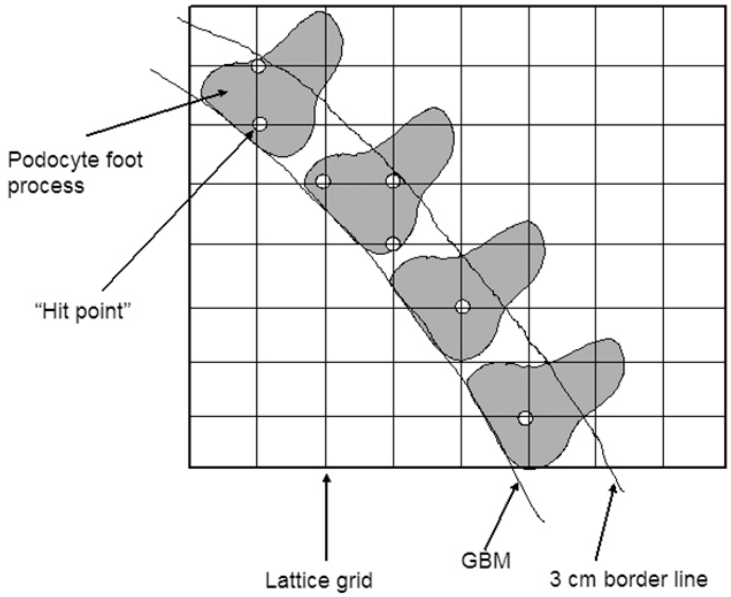

Figure 8. Area measurement on EM micrographs using a transparent lattice grid. Each "hit point" within the 3 cm zone corresponds to an area, which depends on the magnification.

The distance between the GBM and the border line corresponded to 1 µm in Studies I-III and 0,6 µm in Study IV. GBM length was measured with the iTEM software package. The number of slits along the GBM was counted. To describe which gold labels were present in the slit regions, these regions had to be objectively and consistently defined. We used a 1x1 cm square, drawn on a transparent plastic film, which was placed centrally upon each slit on the image, corresponding to ~0.06 μm^2. All gold markers present within this square were defined as "slit-associated".

Based on these measurements, morphology could be described in numbers, and gold labeling could be semiquantified. We used the following terms:

1. Au/μm^2: The number of gold markers per μm^2 foot process cell "area" (which, according to the principles of stereology, is directly proportional to the volume of the cell (125)).
2. Slits/µm GBM: The number of slits divided by GBM length reflects the severity of the foot process effacement in the sample. A value <1 was arbitrarily defined as foot process effacement.
3. Au/slit: the average number of gold particles within each slit area, as defined by the 1x1 cm square.
4. Percentage of Au on slits: This was calculated as the number of markers in the slit areas divided by the total number (i.e. point 3 divided by point 1 above). In the case of slit-associated molecules, this percentage shows whether there is a re-distribution from slit areas to the cytoplasm of the foot processes.

6 RESULTS

6.1 STUDY I (NEPHRIN IN MCNS)

The normal linear fluorescence for nephrin was replaced by a granular one, more pronounced in patients with severe proteinuria. The "granularity" of the IFL was correlated with the degree of foot process effacement observed under the electron microscope; this was taken to reflect the uneven distribution of the foot process effacement (FPE) and longer distances between the remaining slits. Granularity correlated less well with the degree of proteinuria. The semiquantitative immuno-EM revealed a decreased labeling for nephrin in MCNS patients, both in areas with and in areas without foot process effacement (1.07 and 1.42 Au/μm^2, respectively; control 2.79 Au/μm^2). In the visibly diseased areas, nephrin was further reduced, and also redistributed, as only 13% were located in the slit areas compared to 38% in controls and 37% in the normal-looking areas. The amount of nephrin in the slits that were still present was unchanged. We also found a similar reduction of nephrin in one patient with MN (1.18 Au/μm^2) as well as a similar redistribution (unpublished).

6.2 STUDY II (DENDRIN AND ZO-1 IN MCNS)

The IFL and immunoperoxidase stainings showed colocalization of dendrin and nephrin. The staining for dendrin was linear around the capillary loops, and there was no apparent difference between MCNS tissue and controls. Under the electron microscope, dendrin was localized to the slits to approximately the same extent as nephrin (~40%), and slightly apically to ZO-1. Areas with foot process effacement showed a redistribution of dendrin away from the slits. In normal-looking areas there was no change, nor was there any significant decrease in the overall amounts. ZO-1 was redistributed in a similar manner, with no change in the overall amount.

6.3 STUDY III (NEPHRIN, DENDRIN, AND PLEKHH2 IN PAN)

Proteinuria appeared three days after the PA injection and increased thereafter until the peak on day six (Figure 9). To answer our questions regarding early alterations, we chose to study kidney specimens from days 2-4.

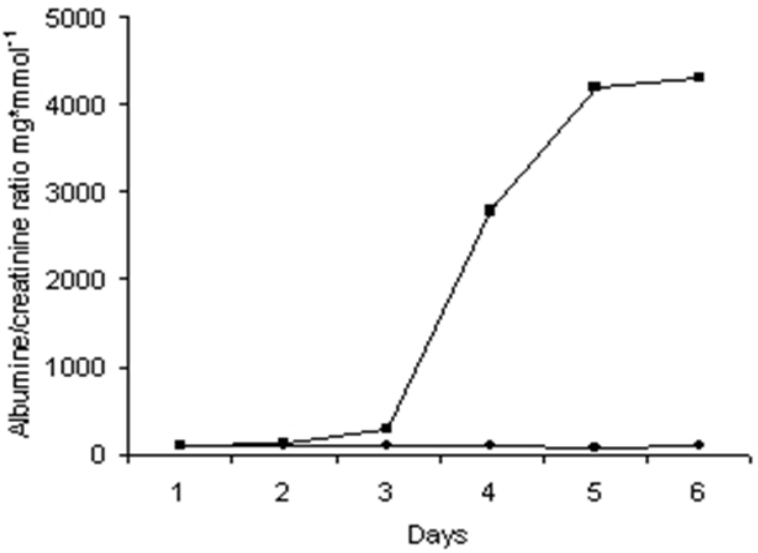

Figure 9. In PAN, proteinuria appeared on day three and continued to increase thereafter.

Under the light microscope, the findings in PAN were mild, with no scleroses visible in the glomeruli. The podocytes contained PAS-positive vacuoles from day three. The cytoplasm of the tubular epithelial cells was filled with protein resorption vesicles. Ultrastructurally, podocytes showed gradually increasing foot process effacement, starting on day two. Some of the slits that were still visible became more dense and "tight junction-like" as the space between adjacent foot process cell membranes diminished. Others looked fairly normal, with visible SDs. Later in the course, starting on day three to four, electron-dense granulae appeared in the podocytes. The shape of the cell membranes changed, with microvilli and large blebs appearing apically. The endothelial cells became swollen. All changes worsened gradually, and by day four, there were no podocytes without foot process effacement, though there were occasional slits that had not undergone this.

IFL showed that dendrin and nephrin, as expected, stained granularly along the capillary loops in PAN on day four in contrast to the normal linear pattern, whereas α-actinin-4 did not show any change.

Semi-quantitative immuno-EM showed decreased expression of nephrin as early as on day two before the animals developed severe proteinuria, both in areas with and without FPE. In contrast, dendrin and plekhh2 were not decreased at this point, though in areas with FPE there was redistribution away from the slits, similar to nephrin. By day four, nephrin decreased even further, as did dendrin and plekhh2. Alpha-actinin-4 was unaffected.

The perfusion experiments revealed damage to the size barrier by day four.

The addition of the ACE inhibitor enalapril did not significantly lower proteinuria, although there was a trend in this direction. Similarly, a blinded review of electron micrographs did not show any ultrastructural differences after enalapril treatment.

6.4 STUDY IV (PDLIM2 IN MN, MCNS, AND FSGS)

In normal kidney tissue, we found pdlim2 exclusively in the glomeruli, using RT-PCR and Western blotting. Light microscopic immunohistochemistry showed a strong linear staining for pdlim2 around glomerular capillaries. Double labeling with nephrin showed overlapping of these two proteins in the podocytes at the light microscopic level. Ultrastructurally, pdlim2 expression was extremely specific for the podocytes, and 98% of the gold particles were found in the foot processes, concentrated in the more central, electron-dense parts. This indicates an association with the actin cytoskeleton. In MCNS and MN, using immuno-EM, the total amount of pdlim2 in podocyte foot processes were significantly lower than in controls, whereas they were unchanged in FSGS.

7 DISCUSSION AND CONCLUSIONS

In order to deepen our understanding of the mechanisms involved in proteinuria, we chose to study renal diseases with nephrotic syndrome with the help of antibodies against proteins already known or suspected to be of importance for an intact glomerular barrier. The choice of diseases can, of course, be discussed. Due to the special embedding technique required for our studies, all samples must be collected prospectively. Therefore, the number of biopsies available was very limited in the beginning. First, we studied MCNS, partly because we had samples available and because it features nephrotic syndrome and foot process effacement without, for example, renal failure or hematuria. MN, in contrast, is characterized by immune deposits. It is the most common cause of nephrotic syndrome in adults, and therefore MN seemed important to study in the next step. FSGS, finally, is difficult to study since there are many subtypes, and not much biopsy material was available to us from each category. Still, it has a poor prognosis, and insights in its pathogenesis would be welcome.

In an experimental model the aim was to elucidate the earliest detectable changes, even before the appearance of proteinuria. For this, the model of choice should be reproducible and predictable. Puromycin aminonucleoside nephrosis (PAN) is such a model, resulting in proteinuria after three to four days. PAN also resembles MCNS in many aspects.

For accurate morphological results, we chose the immuno-EM technique. Among other advantages, this technique offers the possibility to separate normal from diseased areas under the electron microscope, and to make a detailed correlation between protein distribution and the ultrastructural findings. This is important, since both normal areas and pathologically changed areas often can be found in the same

glomerulus in diseased tissues. Foot process effacement, the almost universal finding in proteinuria, is obviously not visible under the light microscope, and Western blots, homogenized glomeruli, and cultured podocytes do not provide morphological information. Only with immuno-EM an integrated view of the subcellular distribution of a certain protein and the cell's morphology can be observed. Furthermore, semiquantification of proteins is less reliable under the light microscope. As pointed out in section 5.4.4, the immunogold EM technique with protein A provides specific labeling 1:1, i.e. one gold marker corresponds to one bound antibody. Moreover, only antigenic sites on the surface of the specimen are available for protein A-gold labeling; another prerequisite for semi-quantification. Immuno-EM specimens are less contrasted than those used for conventional TEM, due to omitting GA and osmium from the preparation. Because of this, to obtain a thorough ultrastructural evaluation, we also performed conventional TEM.

Nephrin is the principal protein of the slit diaphragm bridging the filtration slits between adjacent podocytes. Its key role is highlighted by the dramatic effects in knock-out animals as well as in the congenital nephrotic syndrome of the Finnish type, where nephrin is missing. In acquired human diseases, the role is more obscure. Earlier studies under the light microscope with either immunofluorescence or immunoperoxidase stainings demonstrated that the normal linear staining was replaced by an interrupted and granular one in nephrotic syndrome, irrespective of the type (108-110). To our knowledge, only one immuno-EM study had been conducted so far; it revealed that nephrin gold markers were largely absent in effaced foot processes (110). This is much in line with our own results in Study I, where we performed a semi-quantification of nephrin and could confirm the redistribution. We also found decreased nephrin in areas without visible disease (foot process effacement). This might indicate that a decrease of nephrin protein precedes foot process effacement in MCNS and is not

merely a secondary phenomenon. In the slits that were still present in the MCNS kidney specimens, the nephrin content was equal to that in control slits. It is likely that a certain amount of nephrin protein is required for the formation of a slit, and as soon as it decreases below that level, the SD is dissolved.

In membranous nephropathy, we found an altered nephrin expression comparable to that seen in MCNS (one case). This contradicts the light microscopy findings by Patrakka *et al*. (107) but is more in line with the findings of Doublier *et al.* (108) who also reported a marked decrease in IFL intensity for nephrin in MN. Similar results have also been obtained in the experimental MN analog Heymann nephritis (127). In our MN case, areas without foot process effacement were not analyzed separately, and so further studies would be required to answer the question of whether nephrin is also decreased also in normal areas, as we found in MCNS.

Dendrin, a protein located in the slit area (41, 68), has not previously been investigated in human kidney disease. In Study II we found that dendrin in MCNS is redistributed similarly to nephrin. However, the overall amounts were unchanged and the distribution in areas without foot process effacement was normal. ZO-1 showed a similar pattern, supporting previous studies on ZO-1 under the light microscope (107, 111), which showed no profound changes. Thus, nephrin behaves somewhat differently from dendrin and ZO-1. This might indicate a more important role for nephrin in MCNS, with the redistribution of for example dendrin being a secondary phenomenon. In fact, later studies on dendrin-null mice do not show either proteinuria or foot process effacement (Patrakka *et al.*, unpublished), further supporting this conclusion.

Pdlim2 is associated with the actin-bundling protein α-actinin-4, suggesting a location at a distance from the SD (40). Under the light microscope, pdlim2 overlapped nephrin staining. This observation can be attributed to the low resolution of IFL, since electron

microscopy revealed that pdlim2 markers were clearly concentrated centrally in the foot process, in the electron-dense areas of the actin cytoskeleton. Considering the dramatic change in the shape of the podocyte in nephrotic syndrome (foot process effacement), pdlim2 could play a central role in its pathogenesis. Our studies showed that pdlim2 was equally decreased in MN and MCNS despite the different pathogenic mechanisms. Thus, this decrease could be considered secondary to foot process effacement *per se.* However, in the FSGS group, despite comparable degrees of foot process effacement and proteinuria, pdlim2 expression did not differ from controls. This is in contrast to the findings of others who have found more pronounced alterations in the podocyte-associated molecules (nephrin, podocin,) in FSGS compared to MCNS or MN (109-110). The pronounced differences found in the expression of pdlim2 between MCNS and FSGS could be used in the differential diagnosis of these two conditions, which are sometimes impossible to distinguish from each other histologically. However, in that case, a light microscopical assay should be developed, since immuno-EM is too expensive and time-consuming to be useful in clinical practice. Whether pdlim2 is more involved in the pathogenesis of MN and MCNS than of FSGS, needs further studies, since only a few cases have been studied so far.

As mentioned above, PAN was chosen as experimental model since it is commonly used as a model for MCNS. We chose to study the situation on days 2-4 after the disease induction, in order to capture the early molecular events closely related to the appearance of proteinuria. Nephrin decreased significantly as early as day two, with almost no foot process effacement, nor proteinuria. The perfusion experiments showed damage in the size barrier on day four, in parallel with the appearance of proteinuria, that is, after the change in nephrin expression. Dendrin decreased later and to a lesser extent than nephrin. On day two there was no redistribution of dendrin in areas without foot process effacement, in contrast to the situation of nephrin. Thus, the

sequence of events resembled MCNS in Study II. Plekhh2 is a novel protein of the podocyte, and one that is apparently important for the integrity of the glomerular barrier in zebrafish (Parikka *et al.*, submitted). Under the electron microscope, we found sparse foot process-specific labeling in control rat glomeruli, which decreased to approximately the same extent and at the same time-point as dendrin, that is, on day four in PAN. In conclusion, the earliest changes were seen in the nephrin distribution, both in MCNS and PAN, indicating a central role for the SD, even if the SD and the cytoskeleton are tightly connected both mechanically and functionally. It could be argued that if dendrin were also a part of the SD complex, it would behave similarly. However, even though dendrin has been shown to co-localize with nephrin, dendrin is an intracellular protein and there are no data, to our knowledge, showing that it constitutes a "core" part of the SD itself, like nephrin. The fact that dendrin and plekhh2 behave similarly, despite their different locations and functions, suggests that the changes are secondary to the foot process effacement.

What are the possible implications of the present studies? After the discoveries of nephrin, podocin and other proteins linked to hereditary nephrotic syndrome, there was a need for studies of acquired diseases, which are far more common. It seemed probable that major changes would be found in the expression or distribution of nephrin, dendrin or others in for example MCNS. If so, these molecules could become therapeutic and/or diagnostic targets. The changes which we have found are not dramatic, which suggests that several mechanisms are involved in proteinuria. As stated above, immuno-EM is too costly and time-consuming for routine diagnostic purposes, which is why light microscopic techniques should be developed. However, due to the low resolution, changes in protein expression must be extensive to be visible under the

light microscope. Another problem is that many antibodies fail to bind in paraffin-embedded sections, and/or give a high background signal, due to non-specific binding.

This thesis is a part of a project in which basic genetic research is combined with clinical studies: Genes that are highly expressed in the glomerulus are identified, and their functions are tested in genetically modified mice or zebrafish. Antibodies are raised against the gene products (proteins), and some of them are used in these studies. By putting all this data together, a good picture of the gene of interest can be obtained. For example, plekhh2 (Study III) is necessary for the integrity of the glomerular barrier and the normal glomerular ultrastructure in zebrafish. Conversely, the lack of renal phenotype in dendrin-knockout mice is in line with Study II, where no significant decrease in dendrin expression was seen in human nephrotic syndrome.

It is not likely that glomerular permeability depends on the SD alone. For example, it would be difficult to explain how the proteins that become trapped there are cleared. One would expect them to accumulate at the SD in large amounts, which is not the case. The albumin retrieval hypothesis (i.e. that proteins are indeed filtered and then reabsorbed and metabolized in the tubules), would resolve this particular issue, but is not reasonable for other reasons (see Chapter 1.2.2). Thus, there must be something stopping most of the macromolecules before they reach the SD, most likely the endothelial surface layer; the glycocalyx. For example, pre-eclampsia illustrates the importance of the endothelium; unfortunately, there are no genetic models to test this hypothesis as yet. As we await more research in this field, we should conclude that the glomerular filter needs all its three layers to be intact; the endothelium, the glomerular basement membrane and the podocytes.

8 FUTURE PERSPECTIVES

The collaboration between basic and clinical research can result in new and important discoveries in this field. With the guidance of studies on gene-manipulated animals, we will continue to systematically investigate the expression of potentially interesting glomerular proteins with unknown function, both in patients with nephrotic syndrome and in experimental nephrosis. Hopefully, we will find more clues to the causes of proteinuria and to the pathogenesis of acquired glomerular diseases.

Studies of the expression of novel glomerular proteins in different types of glomerulonephritis may also result in better sub-classification of glomerular diseases with the help of immunohistochemistry. This may in turn help us to identify patients who may, or may not, benefit from aggressive and potentially toxic treatments.

Based on our investigations, further experimental studies are also warranted: In the single shot PAN model, proteinuria resolves after two to three weeks. Studies of protein expression in the healing phase could give further information about the role of different proteins in the pathogenesis of proteinuria.

Finally, looking at the other components of the glomerular barrier would be interesting. For instance, genetically modified animals without a functioning endothelial surface layer would show us a lot about the importance of the endothelium.

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