Karolinska Institutet

From the Department of Clinical Science, Intervention and Technology, Division of Renal Medicine

The expression of the structural proteins Dendrin and Neph1 in the glomerular filtration barrier in proteinuria

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Jenny Hulkko

Karolinska Institutet

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Cover figure: Electron microscopy image of Neph1 (10nm gold) and Nephrin (5 nm gold) in the slit diaphragm of the podocytes.

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TO MIKAEL AND JULIUS

ABSTRACT

Background

In the normal kidney, the glomerular filtration barrier successfully clears about one litre blood per minute. Damage to the filtration barrier might lead to protein leakage in the urine – proteinuria. The major ultra-structural finding in glomerular diseases with proteinuria is foot process effacement (FPE). Despite these being the most common signs of glomerular dysfunction, the underlying pathophysiological mechanisms are still not fully understood. However, a number of proteins identified in the slit diaphragm (SD) of the podocytes, including Dendrin and Neph1, are believed to be significant. Dendrin is a protein that has previously been described in mouse podocytes, associated to the actin cytoskeleton. Neph1 is a transmembrane protein that forms a complex with Nephrin in the SD. Recent studies have indicated the complex involvement in polymerization of the actin cytoskeleton and proteinuria.

Aim

We aimed to study the expression of Dendrin in normal human kidney and in the glomerular disease Minimal Change Nephrotic Syndrome (MCNS) with proteinuria and foot process effacement (FPE). In the second study, we wanted to investigate the subcellular localization of Neph1 in normal human kidney and the expression in Focal Segmental Glomerulosclerosis (FSGS), MCNS and in the corresponding experimental models Adriamycin nephropathy (ADR) and puromycin aminonucleoside (PAN). All characterized by substantial FPE and proteinuria.

Methods

The localization of Dendrin and Neph1 was first studied in normal kidney tissue and then compared to the expression in biopsy specimens of the above mentioned diseases, using light and electron microscopy. The expression was semiquantified by immuno-electron microscopy (iEM).

Results

Dendrin was localized solely in the podocytes close to the SD. There was no significant change in the total amount of Dendrin in MCNS compared to controls by immunofluorescence and iEM. Neph1 was also localized mainly to the SD. Double staining of Neph-1 and Nephrin showed the proteins in close connection in the SD. The total amount of Neph1 was significantly reduced in the glomerular diseases FSGS, MCNS and in ADR and PAN**.** The reduction of Neph1 was also seen in areas without FPE. Nephrin was reduced in MCNS and PAN but unchanged in FSGS.

Conclusion

In preserved slits and in areas without FPE in MCNS, the amounts of Dendrin were unchanged compared to controls. The redistribution might therefore be secondary to FPE. Neph1 co-localize with Nephrin in the SD and was reduced in FSGS, MCNS, ADR and PAN. Nephrin was however, unchanged in FSGS which could indicate a disruption of the Neph1-Nephrin complex and an involvement of Neph1 in the pathogenesis of this disease.

1 LIST OF PUBLICATIONS

I. 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(8):2504-11.

II. **Jenny Hulkko**, Jaakko Patrakka, Mark Lal, Karl Tryggvason, Kjell Hultenby and Annika Wernerson. Neph1 is reduced in primary Focal Segmental Glomerulosclerosis (FSGS), Minimal Change Nephrotic Syndrome (MCNS), and corresponding experimental models adriamycin (ADR) mice and puromycin aminonucleoside (PAN) rats. *Submitted to Nephron.*

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3 LIST OF ABBREVIATIONS

BSA= Bovine Serum Albumin

FPE= Foot Process Effacement

FSGS= Focal Segmental Glomerulosclerosis

GBM= Glomerular Basement Membrane

iEM= Immuno Electron Microscopy

IFL= Immunofluorescence

MCNS= Minimal Change Nephrotic Syndrome

PB= Phosphate buffer

PAS= Periodic-Acid Sciff

PASM= Periodic Acid Silver

SD= Slit Diaphragm

TEM= Transmission Electron Microscopy

4 BACKGROUND

4.1 THE NORMAL KIDNEY

4.1.1 Structure

Structurally the kidney is composed of an outer cortex and an inner medulla (Figure 1). The kidneys functional units are called nephron. A normal human kidney has approximately 1 million nephrons consisting of a glomerulus and a tubular system (Figure 2). The glomerulus consists of small capillary bundles, held together by mesangial cells and their matrix, all encapsulated by Bowmans capsule (Figure 3-5) which is connected to the tubular system.

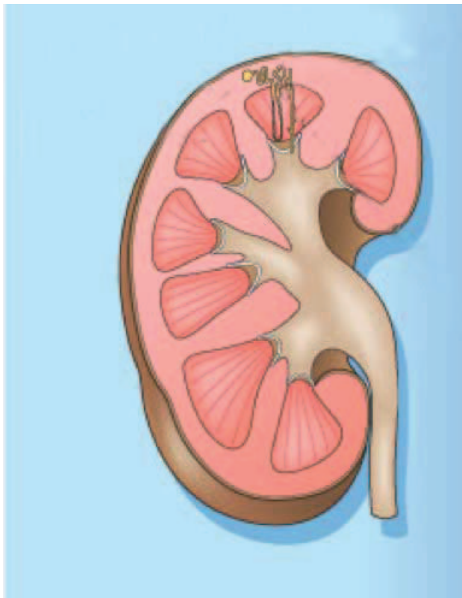

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Figure 1. The normal human kidney. Copyright © 2001, Rights Managed by Nature Publishing Group.

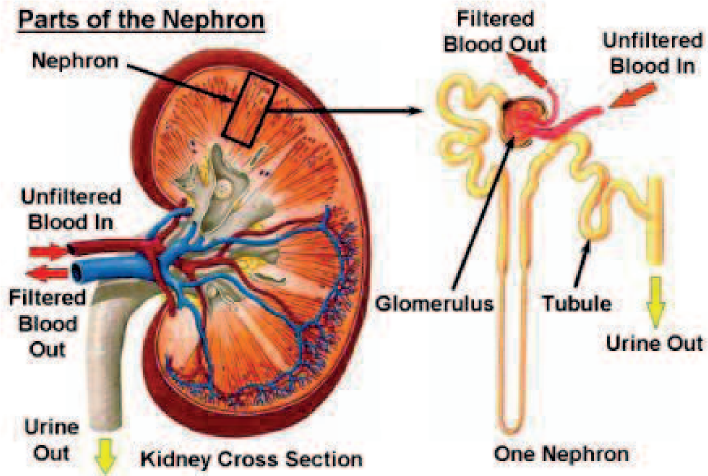

Figure 2. Parts of the nephron. Copyright ©, Rights Managed by unckidneycenter.org

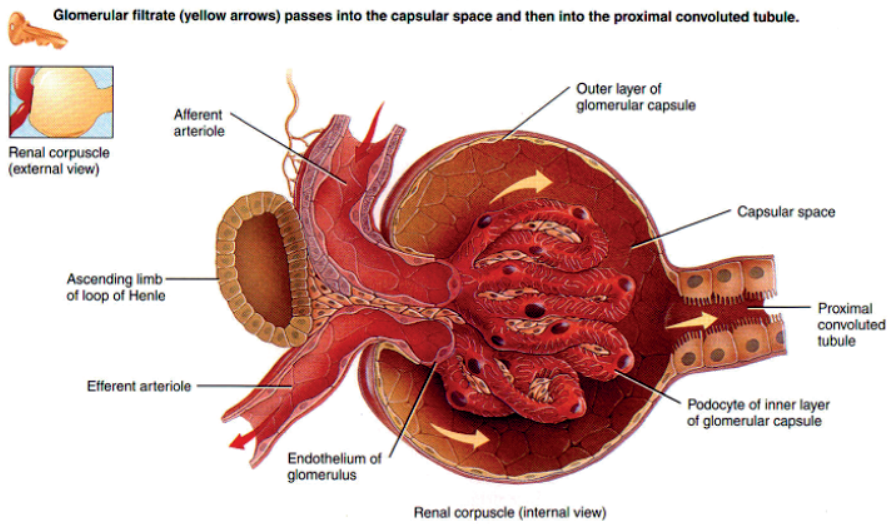

Figure 3. The glomerular filtration. Copyright © 2009, Rights Managed by Nature Publishing Group.

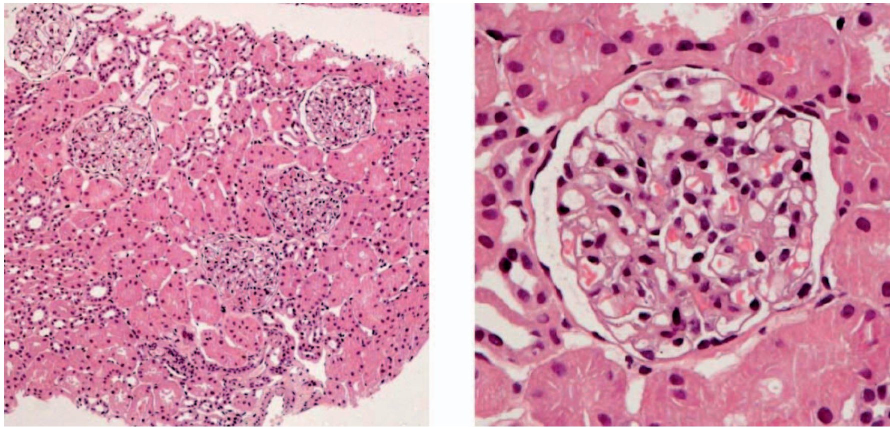

Figure 4a. Light microscopy image of normal glomeruli. Figure 4b. Light microscopy image of normal glomerulus.

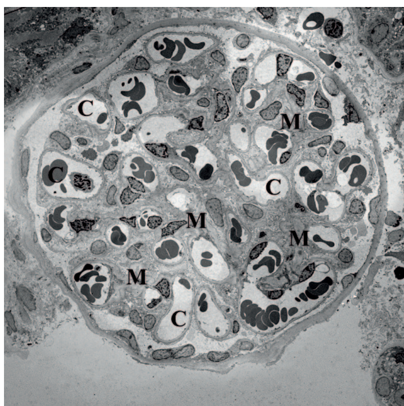

Figure 5. Electron microscopy image of a normal glomerulus. C= capillaries, M=mesangium.

4.1.2 Function

The main function of the kidney is to clear the blood from waste products. This is performed in the glomerulus. Blood enters the glomerulus via the afferent (inward) arteriole. When the blood has been filtered it leaves the glomerulus through the efferent (outgoing) arteriole (Figure 3). The filtration product, the primary urine, is normally free from proteins and blood cells and measures about 180 l/day. Most of this liquid

will be reabsorbed in the tubular system and the amount of urine will be about 1.5 l / day.

4.1.3 Filtration

The filtration barrier of the glomerular capillaries consists of three layers; the endothelium, the glomerular basement membrane (GBM) and the podocytes (Figure 6b, 7). Components of low molecular weight and small size, such as water, urea and glucose pass through the filtration barrier unhindered. Albumin, with its molecular weight of 69kDa, do not pass under normal, healthy conditions. The primary urine is collected in the Bowman´s capsule and will then continue through the tubular system.
The first layer, the fenestred endothelium has a negatively charged cell surface glycocalyx, containing proteoglycans and sialo proteins. It has not historically been considered central to the glomerular perm-selectivity, but growing evidence suggests otherwise (1).
The mayor components of the second layer, the 300-350 nm GBM, are type-IV collagen, negatively charged proteoglycanes, laminin, nidogen and positively charged heparan sulphate. The GBM has been described as the charge-selective barrier for glomerular filtration. This is now questioned by studies on the selective removal of highly anionic substances, which appears to neither influence the glomerular charge selectivity nor increase the degree of proteinuria (2).
The outer layer of the capillary wall consists of podocytes with cell bodies and foot processes (Figure 6b). The foot processes are connected by slit diaphragms (SD) (Figure 6c).

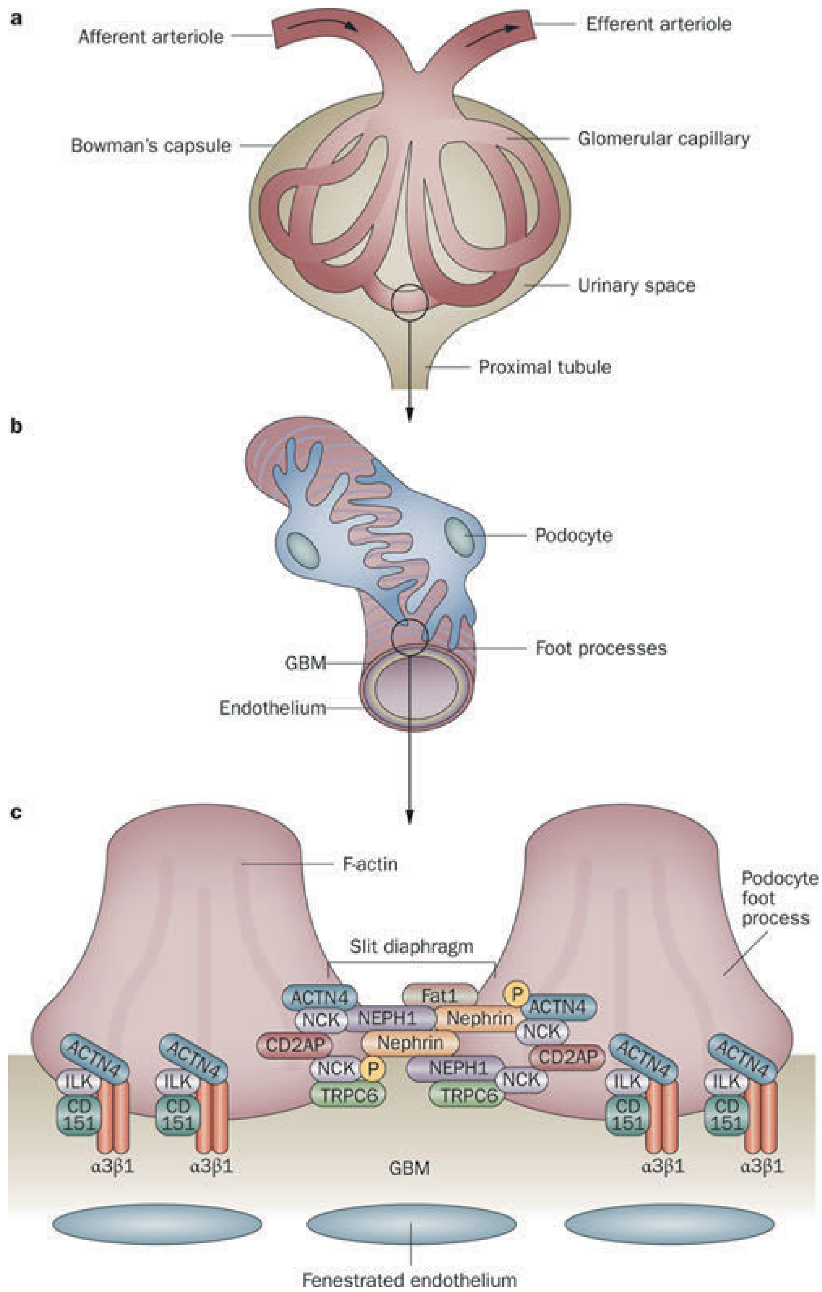

Figure 6. The glomerular capillaries, podocytes and slit diaphragm. Copyright © 2009, Oxford University Press.

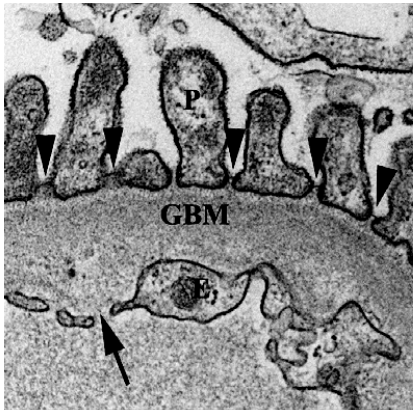

Figure 7. Electron microscopy image of the glomerular filtration barrier. E= endothelium, GBM= glomerular basement membrane, P= podocytes.

4.1.4 The podocyte

The podocyte is a terminally differentiated epithelial cell, which means that damaged is irreversible. This highy specialized cell consists of a cell body, main processes and foot processes. The cell body and the main processes both consists primarily of microtubules and intermediate filaments. The foot processes contain longitudinal actin microfilaments and are anchored to the underlying basement membrane via integrins and dystroglycans. The podocytes both retain the structure of GBM and produce many of its components, for example heparan sulphate proteoglycans. Damage to the podocytes disturbs this symbiosis. A thickening of the GBM due to the podocytes increased production of matrix protein is seen in the glomerular disease Membranous Nephropathy (MN). The podocytes also affect the endothelial cells by production of vascular endothelial growth factor (VEGF) and angiopoetin which act in a paracrine manner on the endothelium (3).

4.1.5 The slit diaphragm

As described above, the foot processes of the podocytes are connected via slit diaphragms, a 25-60 nm wide filtration slit with signaling properties (Figure 6c, 7). Their morphological structure resembles desmosomes and adherens junctions. The

slit diaphragm as a structure was first described by Karnovsky in 1974 in mice and rats (4) and then, the following year in humans (5).
In 1998 Tryggvason et al. identified the protein Nephrin as a major component of the SD. By positional cloning, a mutation in its encoding gene, NPHS1, could be linked to congenital nephrosis of the Finish type (6), a condition characterized by heavy proteinuria in utero. This led to a new interest in the slit diaphragm and their role in glomerular diseases. Since the discovery of Nephrin, numerous new proteins have been identified in the area. It is now recognized that the SD proteins, for example Nephrin (6), P-cadherin (7), Neph1 (8) and FAT (9), are linked to the foot process actin-based cytoskeleton by adaptor proteins (10), such as CD2-associated protein (CD2AP) (11, 12), Zonula Occludens (ZO-1) (13), podocin (14), (Figure 6c) and through the signaling pathways thereby maintain the structure and function of the podocytes (10). The Tryggvason group has in collaboration with our group continued the search for important structural proteins by the usage of knock down zebra fishes and *knock out* mice. The effect on the glomerular filtration barrier is studied with functional tests, light - and electron microscopy (EM) and whether proteinuria is a result of the specific protein being missing or not. Antibodies against the proteins are raised and used to localize the proteins, both by immunofluorescence (IFL) and on a subcellular level in human material by EM.

4.2 PATHOLOGY

Approximately 1 million of the Swedish population suffers from impaired kidney function (15). Globally, the frequency is about 15% (16). Chronic kidney disease leads eventually to renal failure which requires dialysis or transplantation. About 25% of the cases of chronic kidney failure are due to glomerulonephritis (17).
The therapies offered today are unspecific and sometimes even harmful. This is due to the limited knowledge of the mechanisms behind the pathogenesis of acquired glomerular diseases.

4.2.1 Proteinuria and foot process effacement

The most common sign of renal disease and glomerular dysfunction is proteinuria, leakage of proteins, in the urine. This is in itself harmful to the kidney (18). Proteinuria

is caused by the dysfunction of the selective permeability through the filtration barrier, probably foremost affected by structural and functional defects of the podocytes.
The combination of pronounced proteinuria (>3,5 g protein/24h), hypoalbuminuria oedema and hyperlipidemia is referred to as nephrotic syndrome.
A characteristic ultrastructural finding in nephrotic syndrome and proteinuric diseases is so called foot process effacement (FPE) as first described by Farquar in 1957 (19). This shortening and widening of the foot processes is initiated by the re-arrangement of the parallel actin filaments of the cytoskeleton into a compressed network (Figure 8b). However, proteinuria can occur without FPE, and the degree of FPE does not correlate to the amount of proteinuria (20). The underlying cause of FPE is still extensively discussed, as to whether this morphological feature is due to podocytic injury by toxins or infections, a result of protein and/or signaling defects in the SD or cytoskeleton, or altogether secondary to proteinuria.
Indeed, there are several mutations in podocyte-associated proteins identified in hereditary proteinuria, such as CD2AP (11), α-actinin-4 (21), TRPC6 (22) and podocin (23), but it is commonly accepted that proteinuria should not be thought of only as a defect of the podocyte, but rather as a defect of the filtration barrier as a whole, with the lesion in any compartment (3). It is still, however, an open question whether proteinuria precedes FPE or the other way around. Shankland lists the three general views in his 2006 review (24):

1. Effacement in itself causes proteinuria, through a decrease of the number of slit diaphragms along the GBM.
2. Effacement is the result of podocytic injury or abnormality that also leads to proteinuria, but it is not the cause.
3. Effacement and proteinuria are altogether independent of each other.

Shankland places his own vote somewhere in between those.
The following scenarios behind FPE and proteinuria have been singled out by recent podocyte studies, and summarized by Tae-Sun Ha´s 2013 review (25), as:

1. Interference with the SD complex.
2. Interference with the GBM or the podocyte-GBM interaction.
3. Interference with the actin cytoskeleton and its proteins.
4. Interference with the negative apical membrane domain of podocytes.

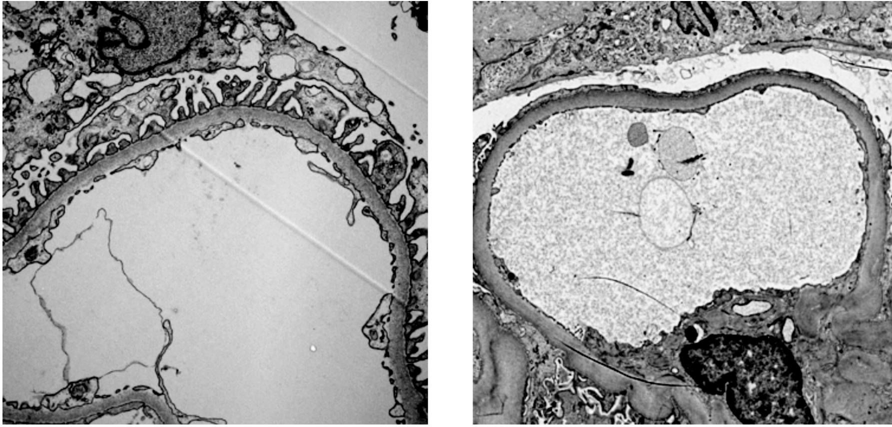

Figure 8a. Electron microscopy image of normal glomerular capillaries. Figure 8b. Electron microscopy image of foot process effacement in MCNS.

4.2.2 Focal Segmental Glomerulosclerosis (FSGS)

One of the most common causes of nephrotic syndrome and a significant cause of chronic renal failure is focal segmental glomerulosclerosis (FSGS). The disease manifests with proteinuria, glomerular lesions of focal and segmental sclerosis and FPE (Figure 9). FSGS can be of either primary or secondary type. Secondary FSGS have an underlying cause such as virus-or drug-association, heritability or can be mediated by adaptive structural-functional responses, such as renal dysplasia or obesity.
The disease is primary a histological diagnosis, based on the presence of segmental sclerotic lesions involving some, but not all glomeruli. Ever since the first portrayals of the disease by Fahr and Rich in 1925 and 1957 respectively, several different types of FSGS have been described (26-28) and numerous attempts of classification have been made. According to the pathologic classification system of Colombia (29, 30), FSGS include five different subclasses based on histological features; cellular, tip lesion, perihilar, collapsing and not otherwise specified (NOS). Based on this classification system, the perihilar type is characterised by sclerosis and/or hyalinosis in the perihilar region of the glomerulus.
The significance of distinguishing between these histological types of FSGS clinically has been debated, but the FSGS studies of Thomas and Deegens, and most recently of D´Agati (31, 32, 33) have demonstrated considerable differences in both clinical features and renal outcome between the histological types.

Recent studies have identified a number of key players in the hereditary form of FSGS, including α-actinin-4 (21, 34), podocin (35), CD2-associated protein (CD2AP) (36), and transient receptor potential channel 6 (TRPC6) (37, 38).

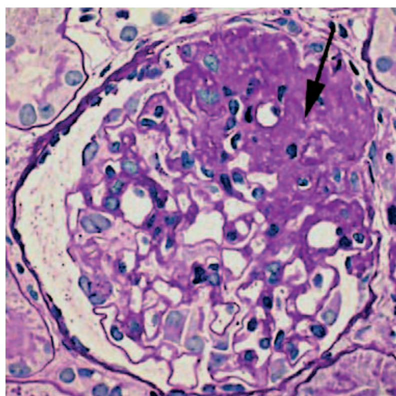

Figure 9. Light microscopy image of FSGS. PAS staining of a glomerulus with a segmental sclerosis (Arrow).

4.2.3 Minimal Change Nephrotic Syndrome (MCNS)

MCNS shares its most prominent clinical manifestations with FSGS, such as severe FPE and proteinuria – however, with normal appearance in light microscopy, as opposed to the focal, segmental glomerulosclerosis that characterizes FSGS. Thereof the name *Minimal Change* Nephrotic Syndrome. The use of transmission electron microscopy (TEM) to identify FPE is therefore a helpful tool in analyzing biopsy material (Figure 8b).

MCNS is the most common disease underlying nephrotic range proteinuria among children. A common belief is that the cause of the podocytic injury in the disease is an abnormal regulation of a T cell subset (39). In 2003, we presented reduced Nephrin expression in the podocytes of MCNS patients (40).

4.2.4 Animal models for proteinuria

Animal models are an indispensable tool for studying the mechanisms of proteinuria and the function of specific proteins of the filtration barrier. Above all, they enable studies of different stages of proteinuria and FPE, from the onset and early stages, to fully developed disease. Based upon the area of interest there are now a vast number of

different animal models available, for example Heymann's nephritis with formation of in situ immune complex, models of hyperfiltration due to reduced number of nephrons and the neutralization of the podocytes glycocalyx that results in FPE (41). We have used two non-transgenic animal models of nephrotic syndrome, puromycin aminonucleoside (PAN) administration to rats, and adriamycin administration (ADR) to mice. The podocytes are injured by toxins in both models. Puromycin interrupts the ribosomal protein translation and adriamycin leads to DNA inserts, resulting in massive proteinuria.

5 AIM

A genomic-, proteomic-, and bioinformatic research program has been launched by groups at KI and KTH to characterize novel glomerular proteins of possible importance in human glomerular disease. By collaboration with these groups, we have access to antibodies raised against newly identified glomerular proteins. The specifically produced antibodies in combination with the biopsy material from the hospitals bio bank offers an unique possibility to study the expression of novel proteins in the renal filtration barrier and their role in the pathogenesis of human glomerular diseases. With the hypothesis that the molecular structure of the podocyte slit diaphragm is altered in proteinuric diseases with foot process effacement we aimed to determine the distribution and localization of cytoskeleton associated proteins. Thus, we chose to study the SD proteins Dendrin and Neph1, both of unknown function, in the normal kidney and their expression in acquired glomerular diseases.

In the Dendrin study we used MCNS as disease material and in the Neph1 study we added FSGS in order to compare the protein expression in a glomerular disease which often leads to chronic renal failure, in contrast to MCNS which is reversible.

6 ETHICAL CONSIDERATIONS

Normal renal tissue was taken from unaffected kidneys that had been surgically removed because of localized carcinoma.

All biopsies were taken for diagnostic purposes and re-examined to confirm the diagnosis. The material is used in agreement with the local ethical board (Dnr 1395–32/2005).

All animal experiment were conducted according to Swedish animal research regulations and approved by the local Board of Ethics (D228/99, 1395-32/2005, S65/2001, S25/2002, S209/03 N250/11).

PRESENTATION OF THE STUDY

This thesis is composed of two articles, one published and one submitted.

The first study describes the cytosolic podocyte protein Dendrin, which has previously been identified in the brain and in mouse podocytes, associated to the actin cytoskeleton. As the regulation of the actin cytoskeleton architecture in the podocyte foot processes is of considerable interest in glomerular diseases with proteinuria and FPE we wanted to study the expression of Dendrin in MCNS. The expression of the protein Zona Occludens-1 (ZO-1), known to be localized close to the SD, was also included as a comparison.

The second study focus on the transmembrane protein Neph1 that forms a complex with Nephrin in the SD. With recent publications (42, 43) indicating the Neph1-Nephrin complex involvement in polymerization of the actin cytoskeleton and in proteinuria, we wanted to study the expression of Neph1 and compare it to that of Nephrin in FSGS and MCNS, and in their corresponding experimental models Adriamycin Nephropathy (ADR) and Puromycin aminonucleoside (PAN)-induced nephrosis.

7 METHODS AND MATERIAL

7.1.1 Human, normal renal tissue

Normal renal tissue was taken from the unaffected parts of kidneys surgically removed because of localized carcinoma.

7.1.2 Patients

Patients, who fulfilled the criteria for FSGS and MCNS clinically and histopathologically, were chosen for the studies. Classification of FSGS was performed according to the Columbia system (29, 30). We used the perihilar variant as it is the most common type of primary FSGS in the biopsy material from our area.

Biopsy material
All biopsies included in the studies were taken for diagnostic purposes. They were all re-examined to confirm the diagnosis. For the iEM studies biopsy material has been saved prospectively and embedded in a low-temperature resin (K11M).

7.1.3 Adriamycin Nephropathy (ADR)

ADR is a well-established experimental model used as an animal model of human FSGS (41). The administration of the toxin adriamycin leads to a progressive, chronic glomerular disease, characterized by proteinuria, segmental glomerulosclerosis, FPE and tubulointerstitial fibrosis. Formerly being a model based upon rats, Wang et al published a protocol for mice in 2000. The method has greatly enhanced the understanding of the processes underlying the progression of renal injury (44).
In our material we measured extensive albuminuria day 7 after injection, along with partial FPE. By day 14 segmental sclerotic lesions were detected in the glomeruli from all animals except one. This material abled us to study the protein expression in the initial phase, as well as in fully developed disease with sclerotic lesions in the glomeruli.

7.1.4 Puromycin aminonucleoside nephropathy (PAN)

Puromycin aminonucleoside is an antibiotic originating from the Streptomyces alboniger bacterium. Intravenous administration of the toxin results in proteinuria and FPE (45), but in contrast to ADR it does not give rise to segmental glomerular sclerosis until after 16 weeks, and is therefore used as an experimental model of MCNS.
In our material, partial FPE was seen by day 2 after injection, before the onset of proteinuria by day 4. By day 4 the FPE was widespread. No sclerotic lesions were present in the glomeruli (46).
We used material from day 2 in order to study the protein expression before proteinuria and the material from day 4 with both proteinuria and FPE as a comparison to human MCNS.

7.1.5 Primary antibodies

In preparation of polyclonal antibodies, the specific protein is biochemically isolated and injected in the animal. The immune response from the B cells gives a large amount of antibodies directed towards *different* epitopes of the antigen, i.e. polyclonal antibodies.The lgG fraction is then usually filtered out for production. To produce monoclonal antibodies, *one (1)* specific B cell is isolated and cloned to produce antibodies directed against the same, specific, epitope.
For immunohistochemistry (especially iEM) studies, polyclonal antibodies are preferred as the probability of one specific epitope, being exposed on the surface of the embedded tissue is very low. If the antibodies are directed against a number of epitopes, the probability of them binding the specific protein increases.

7.2 TISSUE PREPARATION

7.2.1 Tissue preparation for light microscopy

Specimens were fixed in 4% phosphate buffered formaline, dehydrated and embedded in paraffin according to standard procedures. 1,5 µm sections were cut on a microtome, and stained with hematoxylin-eosin, periodic-acid Sciff (PAS), Ladewig trichrome, and periodic acid silver (PASM).

7.2.2 Immunofluorescence (IFL)

An unfixed piece of a renal biopsy taken for diagnostic purposes is snap frozen. 5 µm thick cryosections are incubated with FITC-conjugated antibodies against lgG, lgA, lgM, fibrinogen, the light chains kappa and beta and the complement factors C3 and C1q.
For our studies the cryosections were postfixed with cold acetone (−20°C) and blocked in 5% normal goat serum. Primary antibodies were incubated overnight at 4°C and the secondary fluorescent antibody for an hour in the dark, in room temperature.

7.2.3 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. 10% milk was used as blocking for 30 minutes. The primary antibody, or normal lgG as negative control, was incubated overnight after which the HRP-conjugated secondary antibody (Envision TM, Dako, Glostrup, Denmark) was added for 30 minutes at room temperature and then visualized by the DAB/ H_2O_2 substrate. Nuclei were stained by hematoxylin.

7.2.4 Tissue preparation for transmission electron microscopy

Tissue were fixed in a buffered 2% glutaraldehyde, and post-fixed in osmium tetraoxide (O_SO_4) in order to enhance contrasts. After alcohol dehydration the specimen were embedded in a hard plastic resin to enable the ultrathin sectioning of approximately 40- 60nm. The specimen were then mounted on a grid and finally contrasted by adding the heavy metals, lead citrate and uranyl acetate.

3.2.4 Immuno Electron Microscopy

Small pieces of tissue were dehydrated in methanol and embedded in Lowicryl K11M (Chemische Werke Lowi, GmbH, Waldkreiburg, Germany), a special plastic which

enables iEM. Ultrathin sections were mounted on carbon/formvar nickel grids and incubated in 2% bovine serum albumin (BSA) and 2% gelatin in 0.1 M phosphate buffer (PB) at pH 7.4 followed by incubation overnight with primary antibodies (Dendrin, Neph1 and Nephrin), diluted 1:100 and 1:50 respectively, in 0.1M PB containing 0.1% BSA and 0.1% gelatin (PBBG). Bound antibodies were detected by protein A conjugated with 10 nm colloidal gold (Biocell Laboratories Inc., Rancho Dominguez, CA, USA), diluted 1:100 in PBBG.
In double staining of Neph1 and Nephrin, Nephrin was incubated overnight, followed by Neph1 for 3 hours. Secondary antibodies were conjugated with 5 nm and 10 nm gold particles respectively.

7.2.5 Semiquantification of structural proteins by iEM

The primary antibody is bound 1:1 by protein A molecules tagged with a gold particle which, due to its high density, allows visualisation by TEM. Protein A derives from Stafylococcus Aureus and is used because of its specificity against the lgGs Fc part. This specific binding both enables semiquantification and gives lower background than secondary antibodies which bind to multiple epitopes of lgG.
Sections were examined in a Tecnai 10 microscope (Fei Company, the Netherlands) and digital images are taken by Megaview III (SiS Company, Münster, Germany).

Figure 10. Semiquantification by iEM. Six locations of the glomerulus were chosen according to a random systematic sampling procedure.

Six locations per glomerulus (1–2 glomeruli, depending on material) were chosen with a random start at low magnification along the glomerular capillaries. Three images were

systematically taken from each location, including areas with or without foot process effacement (no sclerotic areas), giving a minimum of 18 images/specimen (Figure 10). Prints at a final magnification of 52 000× were examined and the number of gold markers (Au) counted in the podocytes, GBM and endothelial cells, respectively. The area of corresponding compartment is calculated by point counting, using a 1 × 1 cm square lattice (47), and expressed as μm^2. Dividing the total number of gold particles by the area, give the concentration ($Au/\mu m^2$).

In order to distinguish between areas with and without FPE, the length of the GBM is measured and the number of slits/μm GBM calculated. Less than 1 slit/μm GBM is defined as FPE.

7.3 DATA COLLECTION AND STATISTICS

The material prepared for iEM must be saved and embedded prospectively. Thus, such material can only be prepared if the biopsy is large enough to still allow a proper histopathologic examination. This means that for material from a certain disease we must both rely on the biopsy quality, the amount of material from the biopsy, and the general prevalence of the specific disease in our area. The relatively small number of cases from each specific disease can unfortunately be rather troublesome when it comes to statistics.

8 RESULTS

8.1 MAIN FINDINGS STUDY 1

In normal renal tissue, IFL showed Dendrin in almost complete overlap with Nephrin in a linear pattern along the glomerular capillaries. With EM we could localize Dendrin close to the SD.

Neither the staining pattern nor the intensity of Dendrin or ZO-1 differed between MCNS and controls using IFL, which was confirmed by iEM. No significant change in the total amount of Dendrin or ZO-1 was seen in MCNS compared to controls, neither in FPE areas nor without (Table 1). The proteins were redistributed from the SD in FPE areas to the podocyte cytoplasm. We did not find Dendrin to be translocated to the nucleus in neither PAN (Table 2) nor MCNS (data not published).

Dendrin expression in the podocytes in human kidney

	Slits/µm	**Au/µm^2**	**Au/slit**	**Percentage of Au on slits**
Controls (n=5)	**1,75 ± 0,26**	**1,24 ± 0,39**	**0,10 ± 0,03**	**36 ± 12**
MCNS, areas with FPE (n=5)	**0,52 ± 0,11**	**0,94 ± 0,31**	**0,13 ± 0,09**	**17 ± 9***
MCNS, areas without FPE (n=5)	**1,64 ± 0,27**	**1,51 ± 0,96**	**0,18 ± 0,11**	**32 ± 4**

*Table 1. Expression of Dendrin in controls and MCNS based on immunoelectron microscopy, gold particles/µm^2 (Au/µm^2). The data are presented as mean ± sd, * = $p < 0.05$ compared to controls.*

Dendrin expression in the podocyte nucleus in rat

Controls	**1,18 ± 0,3**
PAN	**1,34 ± 0,5**

Table 2. Expression of Dendrin in controls and MCNS based on immunoelectron microscopy, gold particles/µm² (Au/µm²). The data are presented as mean ± sd.

8.2 MAIN FINDINGS STUDY 2

With iEM we localized Neph1 mainly to the SD in normal human, rat and mice kidney. Double iEM staining of Neph1 and Nephrin showed the proteins in close connection in the SD. This is, to our knowledge the first published EM image of the co-localization, and of Neph1 in human kidney (cover figure).
By iEM semiquantification of biopsies from patients with perihilar FSGS and MCNS, we found a significantly reduced amount of Neph1 in the podocytes both in areas with and without FPE (Table 3). This reduction was also seen in the corresponding experimental models PAN and ADR (Table 4). Nephrin was also reduced in MCNS and PAN but unchanged in FSGS, both in areas with and without FPE (Table 4).

Expression of Neph1 in podocytes in human kidney

	Proteinuria (g/24h)	**In podocyte (Au/µm²)**	**FPE (Au/µm²)**	**Non-FPE areas (Au/µm²).**
Controls (n=5)	**0**	**1,5 ± 0,2**	**-**	**-**
FSGS (n=5)	**1 g/24 h- 8 g/24 h.**	**0,3 ± 0,1***	**0,3 ± 0,2***	**0,5 ± 0,3***
MCNS (n=5)	**1g/l – 27g/24 h**	**0,5 ± 0,1***	**0,5 ± 0,1***	**0,5 ± 0,2***

*Table 3. Expression of Neph1 in controls, FSGS and MCNS based on immunoelectron microscopy, gold particles/µm² (Au/µm²). The data are presented as mean ± sd, * = $p < 0.05$ compared to controls.*

Summary of Neph1 findings

	Neph1	Nephrin
FSGS	↓	→
FSGS, FPE	↓	→
FSGS, Non FPE	↓	→
MCNS	↓	↓
MCNS, FPE	↓	↓
MCNS, Non FPE	↓	↓
ADR, Day 7	↓	→
ADR, Day 7, FPE	↓	→
ADR, Day 7, Non FPE	↓	→
ADR, Day 14	↓	↓
ADR, Day 14, FPE	↓	→
ADR, Day 14, Non FPE	↓	→
PAN, Day 2	↓	↓*
PAN, Day 2, FPE	→	↓*
PAN, Day 2, Non FPE	↓	↓*
PAN, Day 4	↓	↓*
PAN, Day 4, FPE	↓	↓*
PAN, Day 4, non FPE	↓	↓*

Table 4. Summary of findings based on immunoelectron microscopy.
Notes: ↓ = significantly reduced ($p < 0,05$), → = no change compared to controls.
**= Previously published (40).*

9 DISCUSSION

9.1 REFLECTIONS ON METHODS AND RESULTS

9.1.1 Strengths/ Weaknesses of methods

When studying protein expression in the filtration barrier, EM has the great advantage over light microscopy that it can be used to distinguish between areas with and without FPE. This means that the amount of protein can be evaluated in normal and effaced areas, respectively.
The iEM technique that we have developed to semiquantify the expression of proteins is robust and well established at our department. However, due to the limited amount of specially embedded material there are sometimes statistical difficulties, as mentioned before.
The experimental models of PAN and ADR have great advantages when evaluating changes in protein expression in relation to proteinuria and FPE. Though, we are aware of the caution that needs to be held against these models as representations of human glomerular diseases. Mainly because the toxic injury of the podocytes in these experimental models is both nonspecific and acute which affect the molecular composition of the filtration barrier differently than in the slow chronic progression of MCNS and FSGS (41, 48).

9.1.2 Study 1

With no change in the total amount of Dendrin or ZO-1 neither in areas with nor without FPE, the proteins are probably being redistributed from the SD to the podocyte cytoplasm, secondary to FPE. This pattern differs from our results on Nephrin and Neph1 in MCNS, which both are reduced in areas with FPE as well as in areas without, and therefore would be more probable key players in the processes behind FPE.

Recent years studies of Dendrin have focused on the proteins pro-apoptotic signaling properties, and the fact that it seems to accumulate in the podocyte nucleus in response to glomerular injury.

In 2011 Asanuma K et al (49) showed an accumulation of Dendrin in podocyte nuclei in ADR mice and in patients with FSGS, MN and Lupus Nephritis. In line with our results, they did not however, find nuclear Dendrin in MCNS patients.
Shankland, Mundel and colleagues have suggested that podocyte apoptosis causes podocyte loss from the GBM and leads to glomerulosclerosis in human glomerulopathy (3, 50). Asanuma K et al proposed that the relocation of Dendrin to the podocyte nucleus could serve as a marker of disease activity and prediction of progression to glomerulosclerosis in biopsy material. Kodama F et al demonstrated a similar translocation of Dendrin to the podocyte nuclei in patients with lgA nephropathy (IgAN). They concluded that this translocation enhances podocyte apoptosis in acute glomerular injury and leads to podocytopenia in IgAN patients. (51). It would be interesting to continue our Dendrin study with lgA patients in order to examine these findings of nuclear translocation.

9.1.3 Study 2

The reduction of Neph1 in combination with unchanged amounts of Nephrin in FSGS, could indicate a dissociation of the proteins and a disturbance of the complex.
The published results on the Nephrin expression in acquired kidney diseases are rather conflicting. Patrakka et al. (52) found no change in Nephrin expression in FSGS, neither in mRNA levels or by immunohistochemistry. Koop et al. (53) have reported the direct opposite, a marked reduction of the Nephrin protein and an increase at mRNA level. This was, however, evaluated from immunohistochemistry and immunofluorescence, with light microscopy. Kim et al. (54) concluded that the expression pattern in proteinuric diseases varies according to the severity of glomerular damage. Also, despite many attempts to portrait a correlation between the expression of Nephrin in glomerular diseases and the degree of proteinuria, the results are highly inconsistent (55).
No other studies than ours, on the expression of Neph1 in human glomerular disease, have yet been published.
We aimed to expand the study of the Neph1- Nephrin complex to double and traditional immunofluorescence staining. Unfortunately, we did not have frozen biopsy material from FSGS patients in our bio bank of sufficient quality. This is a general problem with frozen material which, despite storage in a -70 degrees Celsius freezer,

tend to dehydrate. The material is therefore both very difficult to cut and to incubate, and the result may not be entirely reliable as the antibodies could bind non-specifically. A further complication was that the Neph1 antibody did not bind paraffin embedded material despite numerous different protocols.

We have indications of Neph1 reduction in congenital nephrosis of the Finnish type. But with only two cases available for iEM, we did not include these data in the article.

6 THE POTENTIAL ROLE OF DENDRIN AND NEPH1 IN THE FILTRATION BARRIER

When podocytes are injured they retract or extend their foot processes, detach from the GBM, or die (56, 57). Thus, the frequency of slit diaphragms is reduced, which results in impaired glomerular filtration with proteinuria as a result.

Dendrin is localized to the SD where it binds Nephrin and CD2AP - an adaptor protein that can repress pro-apoptotic TGF-β signaling (58). Nephrin also binds Neph1 and Podocin in a transmembrane receptor complex, which interacts with actin associated proteins, for example CD2AP, ZO-1 and α-actinin-4. Loss of any of the proteins of the complex leads to proteinuria and FPE as studied in gene targeted mice (8, 12, 23, 59).

Protamine sulphate (PS) perfusion of rodent kidneys is an animal model that results in immediate FPE which can be reversed by heparin sulphate (60). The knowledge about the trigger behind the actin transformation dynamics is very limited but it has been shown that the Neph1-Nephrin complex is phosphorylated following PS perfusion (61), and that the actin associated protein Cofilin-1, regulated by the complex, is needed for the reconstruction of the cytoskeleton following this injury (62).

Complete balance and function of both structural and signaling proteins is clearly required to maintain the morphology of the foot processes, which are crucial to the function of the filtration barrier. However, the intricately of the proteins interplay with the cytoskeleton to maintain the structure and remodel it after podocyte injury is far from understood.

7 CLINICAL SIGNIFICANCE

The main purpose of this project was to increase the understanding of the kidney filtration barrier and the role of structural glomerular proteins in the normal kidney and in acquired, proteinuric diseases. Increased knowledge about the mechanisms behind proteinuria and foot process effacement and the identification of the proteins involved may sharpen our diagnostic tools in the diagnosis of kidney biopsies. The specific proteins could, for example, ideally, be measured in urine and either serve as markers of disease activity or maybe be used for differential diagnosis between FSGS and MCNS. Glomerular diseases may be better classified or sub-classified based on specific, individual protein expression or expression pattern and more directed therapies and thereby less harmful, may be possible in the future.

8 UNPUBLISHED RESULTS AND POSSIBLE FUTURE STUDIES

The most desirable trail to follow in order to find important proteins in glomerular disease would be transcriptome methods to identify up-and down regulated genes in biopsy material. Then continue with studies on knock down zebra fish, an experimental model that is both inexpensive, easy to breed and quickly examined. The knock down effect on the glomerular filtration barrier could be observed by EM and it can also be evaluated whether the zebra fish get proteinuria as a result of the knock down or not. These methods would hint which proteins that would be candidates to further study on human material using light and electron microscopy.

Unfortunately the material from biopsy material is very limited, as mentioned before, which complicates transcriptome studies. Also, not all proteins work in the zebra fish experimental model.

Another way to find new proteins specifically expressed in the glomerulus is through *Human Protein Atlas*, order as oligo products and run on PCR and agarose gel. This way we singled out the protein Prex2 as expressed solitary in the glomerular fraction. After western blot, the antibody was tested on paraffin embedded material and for electron microscopy and protocols were formulated. With iEM, Prex2 could be localized to the cytoplasm of the podocyte foot processes. It has not yet been studied in glomerular disease. A zebra fish knock down has been discussed.

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