Pediatric Graduate School
Hospital for Children and Adolescents and Biomedicum Helsinki
University of Helsinki
Finland

Pathophysiology of Congenital Nephrotic Syndrome of the Finnish type

Arvi-Matti Kuusniemi

ACADEMIC DISSERTATION

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Supervised by: Docent Hannu Jalanko
Hospital for Children and Adolescents
University of Helsinki
Helsinki, Finland

Reviewed by: Docent Heikki Helin
Division of Pathology, HUSLAB
Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Docent Petri Koskinen
Department of Medicine, Division of Nephrology
Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Official opponent: Professor Eero Mervaala
Department of Pharmacology and Toxicology
University of Kuopio
Kuopio, Finland

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To my family

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List of original publications

This thesis is based on the following publications:

I. Kuusniemi AM, Merenmies J, Lahdenkari AT, Holmberg C, Salmela K, Karikoski R, Rapola J, Jalanko H. Glomerular sclerosis in kidneys with congenital nephrotic syndrome (NPHS1). Kidney International 70:1423-1431, 2006.

II. Kuusniemi AM, Lapatto R, Holmberg C, Karikoski R, Rapola J, Jalanko H. Kidneys with heavy proteinuria show fibrosis, inflammation, and oxidative stress, but no tubular phenotypic change. Kidney International 68:121-132, 2005.

III. Kuusniemi AM, Kestilä M, Patrakka J, Lahdenkari AT, Ruotsalainen V, Holmberg C, Karikoski R, Salonen R, Tryggvason K, Jalanko H. Tissue expression of nephrin in human and pig. Pediatric Research 55:774-781, 2004.

IV. Kuusniemi AM, Qvist E, Sun Y, Patrakka J, Rönnholm K, Karikoski R, Jalanko H. Plasma exchange and re-transplantation in recurrent nephrosis of patients with congenital nephrotic syndrome of the Finnish type (NPHS1). Transplantation 83:1316-23, 2007.

The publications are referred to in the text by their roman numerals.

Abbreviations

ACE	angiotensin converting enzyme
CD2AP	cluster of differentiation 2 -associated protein
CNF	congenital nephrotic syndrome of the Finnish type
CP	cyclophosphamide
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
GCS	glomerular cross section
GFR	glomerular filtration rate
HE	hematoxylin and eosin
MCP-1	monocyte chemoattractant protein-1
MIB-1	mindbomb homolog 1
MIF	macrophage inhibiting factor
NAP-2	neutrophil activating protein-2
Neph1	nephrin related protein 1
Neph2	nephrin related protein 2
NPHS1	congenital nephrotic syndrome of the Finnish type
NPHS1	the gene encoding for nephrin
NPHS2	the gene encoding for podocin
NS	nephrotic syndrome
PAN	puromycin aminonucleoside nephrosis
PBS	phosphate buffered saline
PE	plasma exchange
PEC	parietal epithelial cell
ROS	reactive oxygen species
SD	slit diaphragm
SEM	scanning electron microscopy
TEC	tubular epithelial cell
TRPC6	canonical transient receptor potential 6
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
TX	transplantation
WT1	Wilms tumor 1
ZO-1	zonula occludens-1
α-SMA	α-smooth muscle actin

Abstract

Congenital nephrotic syndrome of the Finnish type (NPHS1) is an autosomal recessive disease which is highly enriched in the Finnish population. It is caused by mutations in the *NPHS1* gene encoding for nephrin, which is a major component of the glomerular filtration barrier in the kidney. Patients with NPHS1 have heavy proteinuria and nephrotic syndrome (NS) from birth and develop renal fibrosis in early childhood. Renal transplantation (TX) is the only curative treatment for NPHS1. These patients form the largest group of pediatric kidney transplant children in our country.

The NPHS1 kidneys are removed in infancy and they serve as an excellent human material for studies of the pathophysiology of proteinuric kidney diseases. Sustained proteinuria is a major factor leading to end-stage renal failure and understanding this process is crucial for nephrology. In this study we investigated the glomerular and tubulointerstitial changes that occur in the NPHS1 kidneys during infancy as well as the expression of nephrin in non-renal tissues. We also studied the pathology and management of recurrent proteinuria in kidney grafts transplanted to NPHS1 children.

Severe renal lesions evolved in patients with NPHS1 during the first months of life. Glomerular sclerosis developed through progressive mesangial sclerosis, and capillary obliteration was an early consequence of this process. Shrinkage of the glomerular tuft was common, whereas occlusion of tubular opening or protrusion of the glomerular tuft into subepithelial space or through the Bowman's capsule were not detected. Few inflammatory cells were detected in the mesangial area. The glomerular epithelial cells (podocytes) showed severe ultrastructural changes and hypertrophy. Podocyte proliferation and apoptosis were rare, but moderate amounts of podocytes were detached and ended up in the urine. The results showed that endocapillary lesions – not extracapillary lesions, as generally believed – were important for the sclerotic process in the NPHS1 glomeruli.

In the tubulointerstitium, severe lesions developed in NPHS1 kidneys during infancy. Despite heavy proteinuria, tubular epithelial cells (TECs) did not show transition into myofibroblasts. The most abundant chemokines in NPHS1 tissue were neutrophil activating protein-2 (NAP-2), macrophage inhibiting factor (MIF), and monocyte chemoattractant protein-1 (MCP-1). Interstitial inflammation and fibrosis were first detected in the paraglomerular areas and the most abundant inflammatory cells were monocytes/macrophages. Arteries and arterioles showed intimal hypertrophy, but the pericapillary microvasculature remained quite normal. However, excessive oxidative stress was evident in NPHS1 kidneys. The results indicated that TECs were relatively resistant to the heavy tubular protein load.

Nephrin was at first thought to be podocyte specific, but some studies especially in experimental animals have suggested that nephrin might also be expressed in non-renal tissues such as pancreas and central nervous system. The knowledge of nephrin biology is important for the evaluation of nephrin related diseases. In our study, no significant amounts of nephrin protein or mRNA were detected in non-renal tissues of man and pig as studied by immunohistochemistry and *in situ* hybridization. The phenotype analysis of NPHS1 children, who totally lack nephrin, revealed no marked impairment in the

neurological, testicular, or pancreatic function speaking against the idea that nephrin would play an important functional role outside the kidney.

The NPHS1 kidneys do not express nephrin and antibodies against this major glomerular filter protein have been observed in NPHS1 children after renal TX most likely as an immune reaction against a novel antigen. These antibodies have been associated with the development of recurrent NS in the kidney graft of NPHS1 patients. In our study, a third of the NPHS1 patients homozygous for Fin-Major mutation developed recurrent NS in the transplanted graft. Re-transplantations were performed to patients who lost their graft due to recurrent NS and heavy proteinuria immediately developed in all cases. While 73% of the patients had detectable serum anti-nephrin antibodies, the kidney biopsy findings were minimal. Introduction of plasma exchange (PE) to the treatment of recurrent nephroses increased the remission rate from 54% to 89%. If remission was achieved, recurrent NS did not significantly deteriorate the long term graft function.

In conclusion, the results show that the lack of nephrin in podocyte slit diaphragm in NPHS1 kidneys induces progressive mesangial expansion and glomerular capillary obliteration and inflicts interstitial fibrosis, inflammation, and oxidative stress with surprisingly little involvement of the TECs in this process. Nephrin appears to have no clinical significance outside the kidney. Development of antibodies against nephrin seems to be a major cause of recurrent NS in kidney grafts of NPHS1 patients and combined use of PE and cyclophosphamide markedly improved remission rates.

Review of the literature

1. Congenital nephrotic syndrome of the Finnish type (CNF, NPHS1)

Nephrotic syndrome (NS) is a kidney disorder with protein losses into urine (> 3.5 g/day). It is characterized by low levels of proteins in the blood, generalized oedema, and high blood cholesterol levels. NS beginning within the first three months of life is called congenital nephrotic syndrome. One of the most important forms of congenital nephrotic syndrome is NPHS1, which Hallman and co-workers described in 1956 (Hallman et al. 1956). The disease is more common in Finland than anywhere else in the world and it was the first disease of the so-called Finnish disease heritage. All features of the disease are considered to be secondary to the heavy protein losses, and NPHS1 has served as a model disease for investigating both clinical and pathological aspects of NS and proteinuria.

1.1. Genetics

Congenital nephrotic syndrome of the Finnish type (CNF, NPHS1) is a recessively inherited disease caused by mutations in the NPHS1 gene. The gene has a size of 26kb and is located on chromosome 19q13.1. It contains 29 exons and encodes for a nephrin protein (Kestilä et al 1998). Cases of NPHS1 have been described all over the world and over 60 disease-causing mutations of NPHS1 have been identified (Aya, et al. 2000, Beltcheva et al. 2001, Bolk et al. 1999, Gigante et al. 2002, Koziell at al. 2002, Lenkkeri et al. 1999). The disease is highly enriched in the Finnish population and among the Old Mennonites in Lancaster County, Pennsylvania, USA, where the incidence is 1 in 500. In most parts of the world, the disease is very rare. For example, Albright and co-workers (1990) estimated an incidence of 1:50,000 in North America.

About half of the published cases of NPHS1 come from Finland. The incidence of NPHS1 in Finland is one in 8000 live births (Huttunen 1976, Hallman et al. 1956, Norio 1966). There are two main mutations among the Finns, Fin-major and Fin-minor, that account for 97% of the cases (Kestilä et al. 1998). This uniform mutation pattern can be explained by the founder effect (Norio, 2003). About 60% of the Finnish patients are homozygous for the Fin-major mutation, which is a two base pair deletion (121-122 del CT) in exon 2 resulting in frameshift and an early stop codon. The Fin-minor mutation is a nonsense mutation (3325 C→T) in exon 26. Fin-major mutation leads to a truncated nephrin protein of only 90 amino acids and Fin-Minor to a truncated protein of 1109 amino acids.

In contrast to the Finnish patients, most non-Finns have individual mutations. These are deletions, insertions, and splicing, nonsense, and missense mutations spanning the whole gene. Many of the missense mutations are located in exons coding for the Ig-like motifs of nephrin (Gigante et al. 2005). In Mennonites, 1481 delC mutation is common

and leads to a stop codon (Bolk et al. 1999). Most patients from Malta were homozygous for a nonsense mutation R1160X in exon 27 (Koziell at al. 2002).

1.2. Nephrin

The product of the *NPHS1* gene is nephrin, which is a 1241 amino acids long transmembrane cell adhesion protein of the immunoglobulin family with a molecular size of approximately 185 kDa. It consists an extracellular part with eight immunoglobulin-like and one fibronectin type III-like domain, as well as a transmembrane and a cytosolic region (Figure 1). The cytosolic part has no significant homology with other known proteins, but it has nine tyrosine residues, some of which become phosphorylated during ligand binding (Benzing 2004).

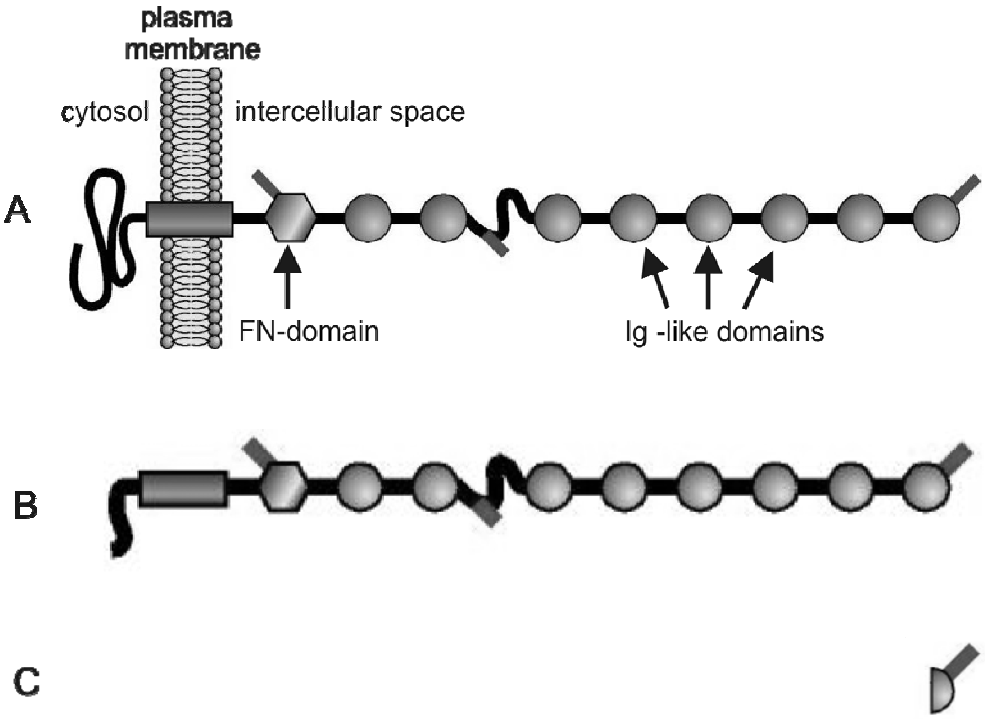


Figure 1 *Nephrin protein. (A) Hypothetical model of the domain composition. Both of the two most common mutations in the Finnish population, (B) Fin-minor and (C) Fin-major, lead to a truncated nephrin protein*

In the kidney, nephrin is synthesized by glomerular epithelial cells (podocytes) (Kestilä et al. 1998), and localized at slit diaphragm (SD) area between the podocyte foot processes (Ruotsalainen et al. 1999). Besides kidney, both nephrin mRNA and protein expression have been reported in the insulin producing ß-cells, in pancreatic islet endothelial cells, and in human pancreas and lymphoid tissues (Palmen et al. 2001, Zanone et al. 2005, Åström et al. 2006). In mice, *NPHS1* promoter activity has been

reported in the brain and pancreas. In the central nervous system, promoter activity was seen in the ventricular zone of the IV ventricle, in the developing spinal cord, medulla oblongata, in the radial glial cells of the cerebellum, hippocampus, and olfactory bulb (Putaala et al. 2000 & 2001, Moeller at al. 2000). Furthermore, nephrin mRNA has also been reported in spleen and thymus and both mRNA and protein in the Sertoli cells of testis in mouse (Liu et al., 2001). In rat, nephrin mRNA and protein have been detected in the follicular dendritic cells of lymphoid tissues (Åström et al. 2006).

A splice variant of nephrin lacking the transmembrane domain has been identified in man. Several splice variants of nephrin have also been found in rat, but their biological significance is unclear (Holthöfer et al. 1999, Ahola et al. 1999). Another interesting finding of unknown significance is an alternative exon 1, termed 1B, which has been found both in man and mouse. It is expressed in mouse brain in the same areas as "normal" nephrin. The transcription of exon 1B leads to nephrin protein with a different amino-terminal end that lacks the typical signal peptide sequence of exon 1A (Beltcheva et al. 2003).

1.3. Podocyte slit diaphragm

SD structure. Nephrin is located at the SD of glomerular podocytes (Figure 2). The SD is not visible in light microscopy, but can be seen in transmission electron microscopy as a filamentous structure bridging the 20-50 nm wide gap between interdigitating foot processes (Yamada 1955, Rodewald and Karnovsky 1974, Furukawa et al. 1991, Ohno et al. 1992). Rodewald and Karnovsky (1974) were the first to suggest a zipper-like organization of this structure. As early as 1972, experiments using large tracer markers suggested that the podocyte SD was a size-selective molecular sieve (Karnovsky and Ainsworth 1972). Furthermore, based on electron microscopic findings, Rodewald and Karnovsky proposed that the SD had an ordered structure with pores smaller than albumin. Direct evidence for the filter function of the SD however was not shown, and knowledge about its molecular composition remained obscure. In 1988, Orikasa and co-workers raised monoclonal antibodies against a rat glomerular extract in mouse. One of these antibodies (named mAb 5-1-6) attached along the surface of foot processes and around SD and caused proteinuria within hours from the injection. The antigen for the antibody remained unknown until the breakthrough in SD biology in 1998 when Kestilä and co-workers isolated the *NPHS1* gene. In 2000, Patrakka and co-workers showed that the most common *NPHS1* gene mutations, Fin-major and Fin-minor, both lead to an absence of nephrin and podocyte SD. This demonstrated both the significance of the SD as a main size selective filter barrier in the kidney and the importance of nephrin to the SD structure.

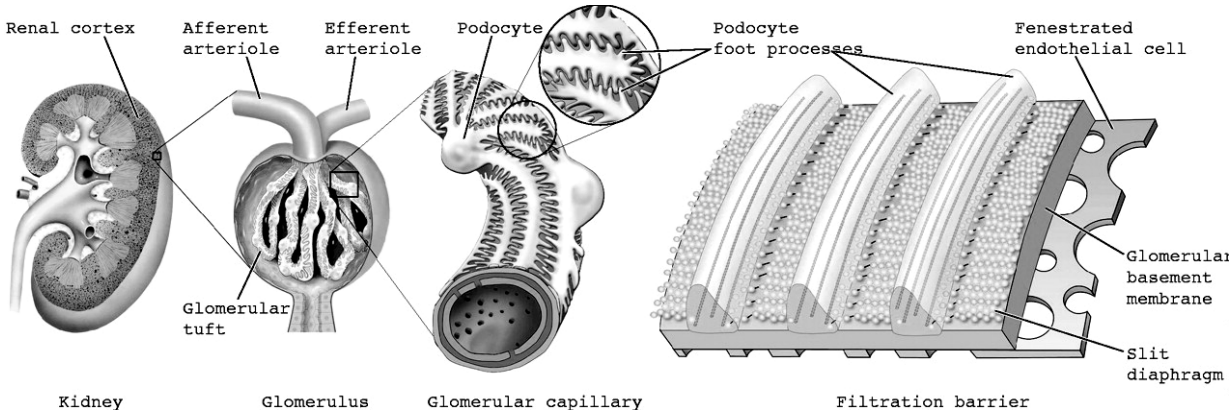


Figure 2 *Location and simplified view of the structure of the slit diaphragm. Reprinted from Tryggvason K, Wartiovaara J: How does the kidney filter plasma? Physiology (Bethesda). 2005; 20:96-101. Used with permission of the copyright holder.*

With new highly advanced electron tomography techniques, it has been possible to visualize the SD structure at a 3- to 5-nm resolution (Wartiovaara et al. 2004). Using this approach, the authors could detect that the molecular strands crossing the filtration slit frequently formed a zipper-like pattern with pores of the size of albumin or smaller located on both sides of a central density. The strands had a similar appearance to that of single nephrin molecules in vitrified solution visualized with the same technique. Wartiovaara and co-workers also localized the distal extracellular nephrin IgG-like motifs close to the center of the slit. These data support the hypothesis that nephrin molecules can interact with each other in the slit and contribute to a SD skeleton of constant width.

SD components. In addition to nephrin, other proteins such as nephrin related protein 1 (Neph1) and 2 (Neph2), FAT1, and FAT2 have been localized to the SD (Table 1, Figure 3) (Inoue et al. 2001, Donoviel et al. 2001, Liu et al. 2003, Gerke et al. 2005, Sun et al. 2005, Cohen et al. 2006). Neph1 and 2 are Ig superfamily proteins like nephrin. Lack of Neph1 causes congenital NS in knock-out mice, but the functional significance of Neph2 is not known (Donoviel et al. 2001). FAT1 and 2 are adhesion proteins and members of the protocadherin superfamily. Absence of FAT1 in mice causes loss of SD, proteinuria, forebrain and ocular defects, and perinatal death (Ciani et al. 2003). Lack of FAT2 causes only proteinuria (Sun et al. 2005).

Table 1. *Some of the most important proteins associated with the slit diaphragm protein complex.*

Protein	Type	Association of the gene defect to renal dysfunction	Reference
Nephrin	TM	AR congenital nephrotic syndrome in man	Kestilä et al. 1998
Neph1	TM	-/- mice have proteinuria and die within 8 weeks of life	Barletta et al. 2003
Neph2	TM	no renal phenotype	Gerke et al. 2005
FAT1	TM	-/- mice have proteinuria and extrarenal defects, and perinatal death	Ciani et al. 2003
FAT2	TM	-/- mice have proteinuria	Sun et al. 2005
VE-cadherin	TM		Cohen et al. 2006
P-cadherin	TM	-/- mice have no renal phenotype	Reiser et al. 2000, Radice et al. 1997
Dendrin	IC	unknown	Patrakka et al. 2007
Densin	TM	unknown	Ahola et al. 2003
Podocin	IM	AR steroid-resistant nephrotic syndrome in man	Boute et al. 2000
CD2AP	IC	-/- mice die of a nephrotic syndrome in 6 weeks, -/+ causes susceptibility for glomerulonephritis in mouse and man	Shih et al. 1999 & 2001, Kim et al. 2003
ZO-1	IC	unknown	Schnabel et al. 1990
MAGI1	IC	unknown	Hirabayashi et al. 2005
MAGI2	IC	unknown	Lehtonen et al. 2005
CASK	IC	unknown	Lehtonen et al. 2004
p120-, α-, β-, γ-catenins	IC	unknown (except: β -catenin -/- early lethality during fetal development in mice)	Reiser et al. 2000, Lehtonen et al. 2004
IQGAP1	IC	unknown	Lehtonen et al. 2005
α-actinin-4	IC	AD slowly progressing FSGS in mouse and man	Drenckhahn and Franke 1988, Kaplan et al. 2000
αII-, βII-spectrin	IC	unknown	Lehtonen et al. 2005

AD; autosomal dominant inheritance, AR; autosomal recessive inheritance, CASK; calcium/calmodulin-dependent serine protein kinase, CD2AP; cluster of differentiation 2 -associated protein, IC; intracellular protein, IQGAP1; IQ motif containing GTPase activating protein 1, IM; integral membrane protein, MAGI1 ; membrane associated guanylate kinase 1, MAGI2 ; membrane associated guanylate kinase 2, TM; transmembrane protein, ZO-1 ; zonula occludens-1.

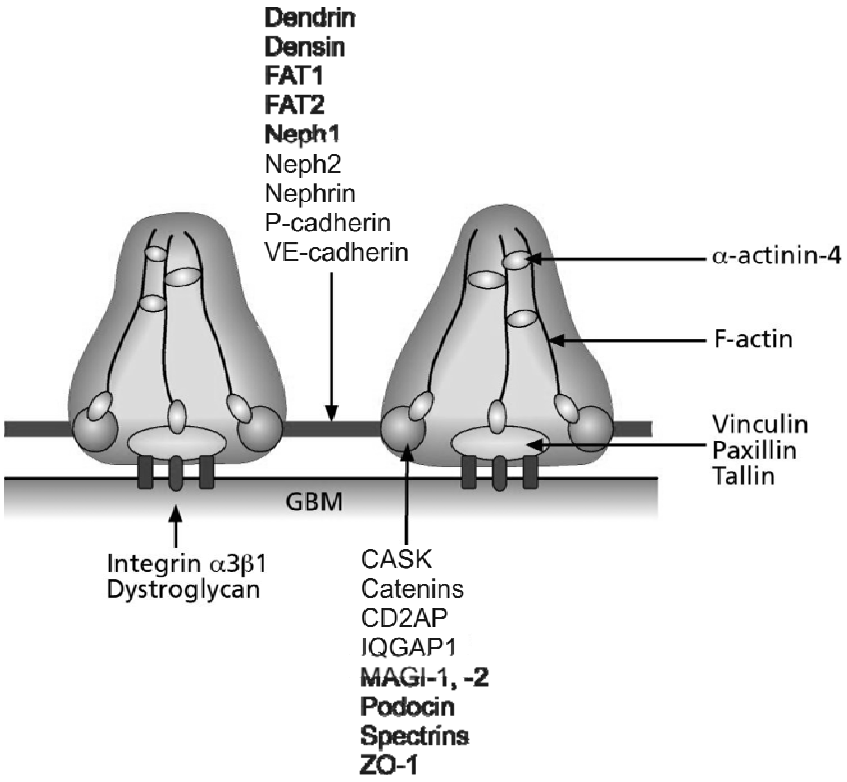


Figure 3 *Schematic drawing of podocyte foot processes and the intervening slit diaphragm (SD). Most of the SD proteins and proteins associated with the SD are mentioned with some of the key proteins of the podocyte cytoskeleton.*

Nephrin, Neph1 and Neph2 contain a short cytoplasmic tail with a number of tyrosine residues that are phosphorylated and engage intracellular signalling events. The viral sarcoma (v-Src) oncogene homolog (Src) family nonreceptor protein tyrosine kinase Fyn directly binds to the cytoplasmic tail of nephrin and mediates nephrin phosphorylation (Verma et al. 2003). Adapter protein Nck and presumably proteins associated with Nck are recruited to phosphorylated nephrin, resulting in Nck-dependent regulation of local actin dynamics (Verma et al. 2006). It is interesting that targeted deletion of Fyn in mice resulted in a severe podocyte dysfunction, foot process effacement, and proteinuria (Verma et al. 2003, Yu et al. 2001). Furthermore, nephrin phosphorylation activates a serine-threonine kinase AKT (Huber et al. 2003a) through activation of a phosphoinositide 3-OH (PI3) kinase. Nephrin-mediated activation of PI3 kinase and the downstream effector AKT has been shown to inhibit podocyte apoptosis and repress collagenase expression and to induce the synthesis of the key components of the glomerular basement membrane; laminin and type IV collagen (Huber et al. 2003a, Li et al. 2001, Mawal-Dewan et al. 2002). In vitro data suggests that nephrin can form heterodimers with Neph1 or Neph2 through their extracellular domains and Neph-mediated interactions may link and stabilize nephrin molecules to induce nephrin-dependent signaling processes (Lahdenperä et al. 2003, Liu et al. 2003, Gerke et al. 2003, Barletta et al. 2003).

Adaptor proteins. Several proteins specifically located in the plasma membrane or to the cytoplasmic side of the SD have been identified (Table 1, Figure 3). Podocin is a hairpin-shaped integral membrane protein with both ends directed into the intracellular space (Boute et al. 2000, Roselli et al. 2002). Podocin interacts with the intracellular domains of nephrin and Neph1 and with cluster of differentiation 2 associated protein (CD2AP) (Sellin et al. 2003, Schwarz et al. 2001). Nephrin-mediated signaling is facilitated by the direct interaction with podocin (Li et al. 2004). Mutations in the podocin gene (*NPHS2*) result in autosomal recessively inherited manner to an early onset childhood proteinuria and focal segmental glomerulosclerosis (FSGS) in humans (Boute et al. 2000). Podocin gene defects are of major importance worldwide as *NPHS2* mutations seem to account for as much as 26% of all steroid-resistant NS in pediatric populations and about 10% of familial FSGS in adults (Weber et al. 2004, Ruf et al. 2004).

Canonical transient receptor potential 6 (TRPC6) is a calcium-permeable cation channel. It is interesting, that TRPC6 channel opening is regulated by the same Fyn kinase that is associated with nephrin (Hisatsune et al., 2004). Mutations in *TRPC6* gene causing FSGS in humans are inherited in an autosomal dominant manner (Reiser et al. 2005). CD2AP is an intracellular protein initially characterized as a T-lymphocyte CD2 adapter protein (Dustin et al. 1998). CD2AP appears to connect the nephrin slit complex with actin modifying proteins (Hutchings et al. 2003, Lynch et al. 2003, Badour et al. 2003). Mice completely lacking CD2AP die of massive proteinuria 6 wk after birth and CD2AP haploinsufficiency seems to be linked to glomerular disease susceptibility both in mice and in humans (Shih et al. 1999, Kim et al. 2003, Wolf and Stahl 2003).

At first it was thought that the SD only forms a passive filtering barrier between podocytes. However, accumulating evidence suggests that SD protein complex does not only serve as static molecular sieve but rather is a highly dynamic functional protein complex (Asanuma and Mundel 2003). Signaling at the SD may be critical for maintaining the integrity of the podocyte architecture and the function of the glomerular filter of the kidney (Huber et al. 2001, 2003a, 2003b, & 2003c). It seems that these proteins are involved in initiating signaling to regulate complex biological programs in the podocyte, such as regulation of cytoskeletal rearrangements, cell differentiation and suppression of proliferation, mechanotransduction, and podocyte viability (Benzing et al. 2004).

SD in renal disease. Besides inherited nephrotic syndromes, SD as a size selective filtration barrier has a significant role in a variety of renal diseases. A reduction in nephrin expression has been shown in patients with diabetic primary acquired nephrotic syndromes, including membranous glomerulonephritis, minimal change glomerulonephritis, and FSGS (Doublier et al. 2001). In HIV nephropathy, a viral product transactivating factor (Tat) induces downregulation of nephrin in podocytes (Doublier et al. 2007). Moreover, a correlation between changes in nephrin expression and proteinuria has been shown in experimental models of glomerulonephritis, such as puromycin aminonucleoside-induced nephrosis and mercury chloride glomerulonephritis (Luimula et al. 2000).

In nephropathic patients with type 1 diabetes, nephrin staining was extensively reduced in renal biopsy specimens (Doublier et al. 2003). Nephrin protein production was also downregulated in the type 2 diabetic subjects compared with the nondiabetic control subjects (Koop et al. 2003, Benigni et al. 2004, Langham et al. 2002), and the decrease in nephrin correlated with the broadening of the foot process widths. In contrast, CD2AP expression was not reduced in podocytes from diabetic patients, suggesting that the reduction in nephrin was not due to widespread podocyte loss or injury (Benigni et al. 2004); this underscores the importance of nephrin in maintaining podocyte integrity. Finally, angiotensin converting enzyme (ACE) inhibitors have been shown to reduce proteinuria and stabilize nephrin expression in type 2 diabetic patients (Langham et al. 2002).

Also, changes in other SD proteins than nephrin have been observed in proteinuria. Podocin, which is normally found in close proximity to nephrin in glomeruli, was clearly dissociated from nephrin in glomeruli of puromycin aminonucleoside nephropathy (PAN) (Kawachi et al. 2003). Recently, Nakatsue and co-workers have shown that both podocin and CD2AP were clearly dissociated from nephrin before the onset of proteinuria in Heymann nephritis (Nakatsue et al. 2005). All of these observations suggest that alteration of the molecular arrangement in the SD is involved in the development of proteinuria in various renal diseases.

1.4. Pathology of NPHS1 kidneys

Macroscopically, the kidneys of NPHS1 patients are 2-3 times larger that those of normal children. In ultrasound scans, cortical echogenity is increased and the corticomedullary border is indistinct (Bratton et al. 1990, Huttunen 1976, Salame et al. 2003). In light microscopy, there are several features indicative to NPHS1, but no pathognomonic findings (Rapola and Savilahti 1971). Characteristic findings include the irregularly dilated proximal and distal tubules with increased number of cytoplasmic vacuoles filled with colloid-like material. The tubular epithelium is flat and atrophic, mesangial cells are hypercellular and the amount of mesangial matrix is increased. However, it seems that glomerular size and number are not significantly increased which is in contrast to what was originally stated (Autio-Harmainen and Rapola 1981). Glomeruli are fibrotic and Bowman's space is dilated. The lesions, except for mesangial hypercellularity, are regarded progressive in nature but possibly secondary to extensive proteinuria (Tryggvason and Kouvalainen 1975, Huttunen et al. 1980, Autio-Harmainen and Rapola 1981, Rapola 1981). Immunofluorescence studies have not revealed IgG or complement component deposits in the NPHS1 kidney glomeruli (Rapola et al. 1992).

Ultrastructurally, the most characteristic finding is the fusion and effacement of foot processes of podocytes (Aula et al 1978, Rapola and Savilahti 1971, Lahdenkari et al. 2004). The same phenomenon is seen in nephrotic syndromes of any cause (Fogo 2003). The width of the interpodocyte slit varies greatly from 20 to 90 nm, and no filamentous image of podocyte SD is seen (Patrakka et al. 2000). Due to foot process effacement, the density of the podocyte slit pores is lower in NPHS1 than in normal kidney (Lahdenkari et

al. 2004). In scanning electron microscopy the podocyte cell bodies are prominent and have a balloon-like appearance. Often only one primary process connects the cell body to a flat cytoplasmic sheet with no branching (Figure 4). Occasional lumpy protrusions of the cytoplasm of foot processes are seen. No areas of denuded glomerular basement membrane (GBM) or holes in the epithelial cell covering are observed (Lahdenkari et al. 2004).

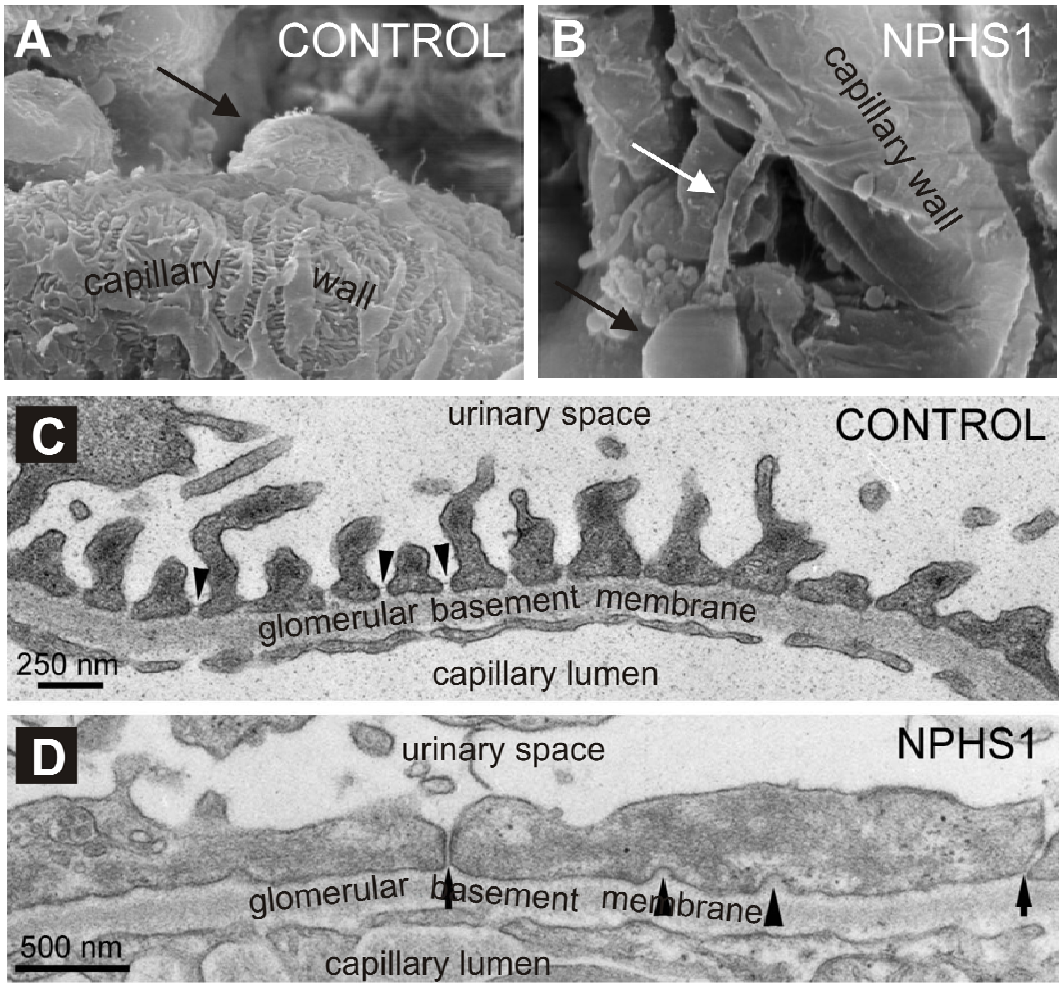


Figure 4 *Scanning and transmission electron microscopic images of the glomerular capillary wall in NPHS1 and normal kidneys. (A) Interdigitating podocyte foot processes are seen in the normal glomerulus. (B) In NPHS1, often only one long primary process (white arrow) connects the cell body to a flat cytoplasmic sheet covering the capillary wall. Black arrows indicate the cell body of the podocyte. (C, D) Flattening of the podocyte foot processes in NPHS1 is also evident in transmission electron microscopy. Adapted from Lahdenkari AT et al: Podocytes are firmly attached to glomerular basement membrane in kidneys with heavy proteinuria. J Am Soc Nephrol. 2004; 15(10):2611-8. Used with permission of the copyright holder.*

1.5. Clinical features

NPHS1 patients are typically born prematurely (<38th week) and the placenta is disproportionately large for an unknown reason, weighing more than 25% of the child's birth weight. Proteinuria begins in utero and can be detected in the first urine sample (Patrakka et al. 2000). The protein loss is severe with serum albumin usually <10 g/l at presentation. Most of the lost protein is albumin, but immunoglobulins and many other proteins, including plasminogen and antitrombin III are also lost. Within a few days, the classical findings of NS, edema and weight gain usually become evident (Huttunen 1976). As a result of the protein losses, the infants are at increased risk for infections and thrombotic complications. Hyperlipidemia is also present as in other nephroses (Holmberg et al. 2004). Infants with NPHS1 do not have any major nonrenal malformations. However, minor functional disorders in the central nervous system and cardiac hypertrophy are common during the nephrotic stage. Most have muscular hypotonia (Jalanko et al. 2007).

1.6. Treatment

Remission cannot be achieved in NPHS1 with immunosuppressive medication. Therefore, before the introduction of renal transplantation (TX), all the Finnish NPHS1 patients died at infancy (Holmberg et al. 1995). As soon as the diagnosis is made, the children are treated intensively with daily albumin infusions (3-4 g/kg/d) and a high-caloric diet (130 kcal/kg/d) to compensate the protein losses. Calcium, magnesium, and thyroxin supplements are given. The medication also includes warfarin to prevent thrombotic complications and prompt antibiotic therapy for septic infections. After a patient reaches a weight of 7kg, bilateral nephrectomy is performed and peritoneal dialysis commenced to further improve the nutritional state (Holmberg et al. 1995, Antikainen et al. 1992). Renal TX is usually performed when a patient has reached a weight of 10kg.

1.7. Long-term outcome

Long-term outcome of NPHS1 children is generally good after renal TX. Qvist and co-workers (1999) reported 5-year patient and graft survival rates of 98% and 82%, respectively. Nearly 80% of the children attend normal school and have normal motor performance. However, the electroencephalogram (EEG) findings are abnormal in 35% of the patients, and some receive anti-convulsive treatment after TX. Sensorineural hearing loss has been documented in 15% of the patients (Qvist et al. 2002). The psychosocial adjustment in NPHS1 children does not differ from healthy children. However, somatic complaints and social problems were reported more frequently in boys, and attention problems in both boys and girls. Furthermore, health-related quality of life (HRQOL) index is significantly lower than measured in healthy controls (Qvist et al. 2004). The most critical times in NPHS1 children's life are the early months since majority of the

disabilities originate before TX (Qvist et al 2003). Most of the children grow well after the transplantation. In a report by Qvist and co-workers (2002), 75% of the NPHS1 children had a height over -2 height standard deviation scores (hSDS) or height velocity was over the 25th percentile and therefore did not need growth hormone treatment.

1.8. Post-transplantation recurrence of nephrosis

After the renal TX, the most severe complication in Finnish NPHS1 patients is recurrence of severe proteinuria (Holmberg et al. 2004). It was first described in 1989 and there are case reports of NPHS1 recurrence worldwide (Sigström et al. 1989, Lane et al. 1991, Flynn et al. 1992, Laine et al. 1993, Kari et al. 1999). Most of the reported cases are from Finland and recently Patrakka and co-workers (2002) described the largest study so far including 15 episodes of recurrent NS in 13 of 51 grafts transplanted to 45 Finnish children. Rescue therapy with cyclophosphamide was successful in seven of the episodes. All Finnish patients with recurrence had Fin-major/Fin-major genotype, which leads to a total absence of nephrin in the native kidney. Half of the patients with recurrence had circulating anti-nephrin antibodies, suggesting that they get immunized against a new antigen found in the transplanted graft, nephrin (Patrakka et al. 2002). Recently Srivastava and co-workers (2006) reported a NPHS1 patient with recurrence that had no auto-antibodies to nephrin, but glomerular permeability to albumin (Palb activity) was increased. It is therefore likely that also other, yet unknown, factors play a role in the recurrence of proteinuria after TX in patients with NPHS1.

2. Proteinuria and renal pathology

Hematuria and proteinuria are cardinal manifestations of renal disease. Proteinuria is a very sensitive indicator of glomerular damage. In a normal adult, the amount of protein excreted in the urine in 24 hours is 80 mg. When this exceeds 150 mg per day, it is abnormal. In NS, there is proteinuria >3.5g per day, hypoproteinemia, hypercholesterolemia, and generalized edema (Glassock 2001). The prevalence of sustained proteinuria in the world is increasing, mostly because of a rapid increase in the incidence of diabetic nephropathy. In 1997, the worldwide prevalence of diabetes was 124 millions, and this number is expected to increase to 221 millions in 2010 (Amos et al. 1997). As a result, in the United States, the incidence of diabetic nephropathy has increased by 150 percent in the past decade (Remuzzi et al. 2002).

2.1. Chronic proteinuria and progression of renal disease

In proteinuric renal diseases, the initial insult to the kidney is usually followed by a progressive decline in GFR (Brenner et al. 1982). More than 80 years ago, Volhard and

Fahr (1914) and Mollendorf and Stohr (1924) detected hyaline droplets in the cytoplasm of proximal tubular epithelial cells (TECs) and considered them as a clear evidence of renal damage related to severe proteinuria. In 1954, Oliver and co-workers (1954) suggested that the appearance of droplets could be a sign of abnormal processing of plasma proteins that is normally carried on by proximal TECs. In the 1970's, it was shown that, among patients with FSGS, mesangiocapillary glomerulonephritis, or membranous nephropathy, those with NS progressed more rapidly to renal failure than those who had never been nephrotic (Cameron et al. 1978, Row et al. 1975). In 1988, Eddy and Michael observed a positive correlation between the degree of albuminuria and the intensity of the interstitial cell infiltrate in a model of NS induced in PAN rats. After that, several other studies in rat models revealed a close link between proteinuria and the subsequent renal structural injury (Remuzzi et al. 1997). The concept of proteinuria driving the progression of renal disease was further supported by animal studies with a different approach. In rats with renal mass ablation (Hostetter et al. 1986), as well as in animals with experimental diabetes (Wen et al. 1985) or adriamycin nephropathy, a low-protein diet, by restoring the size-selective properties of the glomerular barrier, prevented proteinuria and renal injury (Remuzzi et al. 1997).

The first human studies on proteinuria were performed in the 1990's. Proteinuria was a strong and independent predictor of renal outcome in patients with diabetic nephropathy and in patients with nondiabetic renal disease (Breyer et al. 1996, Peterson et al. 1995). The benefits of a low-protein diet in reducing proteinuria and slowing the decline of renal function were shown in patients with insulin-dependent diabetes or nondiabetic proteinuric glomerulopathies (Walker et al. 1989, El Nahas et al. 1984). In addition, pharmacological interventions proved effective. Studies comparing ACE inhibitors with more conventional antihypertensives showed that, at comparable levels of blood pressure reduction, ACE inhibitors more effectively lowered proteinuria and the rate of decline in GFR in diabetic patients (Bjorck et al. 1992, The Gisen Group. 1997).

2.2. Glomerular sclerosis

Glomerular diseases are caused by multiple mechanisms. Progressive glomerular injury is characterized by the development of segmental or global glomerulosclerosis independent of the nature of the underlying renal disease. The progression of chronic renal disease tends to follow a stereotypical course in many cases. Regardless of the nature of the initial insult, once a substantial portion of the renal tissue has been destroyed, there is a steady decline in the glomerular filtration rate with time associated with a progressive loss of viable nephrons. A common histologic finding in these cases is FSGS with tubulointerstitial fibrosis (Ichikawa et al. 1996, Rennke et al. 1994, Klahr et al. 1988). To understand the pathways leading to glomerulosclerosis, it is helpful to distinguish between endocapillary and extracapillary injuries. Endocapillary compartment consists of capillaries and the mesangium. Extracapillary compartment is separated from the endocapillary compartment by the GBM and consists of podocytes, Bowman's space and

Bowman's capsule. The latter comprises the pariethal epithelium and the parietal basement membrane (PBM).

Endocapillary lesions. Initially, glomerular mesangial cell injury was considered to be central to glomerular sclerosis and is a widely studied phenomenon in both experimental animals and human renal diseases. Mesangial cell proliferation with subsequent matrix deposition that leads to glomerular capillary occlusion has been invoked as central mechanism by a number of researchers (Kashgarian et al. 1992, Jacobson et al. 1991, Floege et al. 1992, Couser et al. 1994, Kopp et al. 1993, Striker et al.1989, Egido et al. 1996, D'Amico et al. 1995). In experimental glomerulopathies, the most common endocapillary injury is mesangiolysis with various degrees of endothelial involvement, such as, in Thy-1 mediated nephropathy (Johnson et al. 1994) or in Masugi nephritis (Shirato et al. 1996, Kühn et al. 1977). These endocapillary injuries are subject to proliferative repair with subsequent apoptosis of the surplus daughter mesangial cells (Baker et al. 1994, Savill et al. 1995, Hugo et al. 1996) as well as endothelial cells (Shimizu et al. 1996, Choi et al. 1995). Despite this, the native structure of the mesangium cannot always be fully restored. A common opinion is that areas of solidified mesangial expansion (mesangial sclerosis) are successfully healed by scarring, that is, successful in the sense that the supporting function of the mesangium has been reestablished with reconnection of the GBM to the mesangium. These areas appear fairly stable, and progression to segmental sclerosis has not been demonstrated in experimental settings (Kriz et al. 1998a). Therefore, based on current knowledge, pure mesangial injury (typically observed in rat anti-Thy1 nephropathy) causes only reversible changes but not glomerular sclerosis (Mauer et al. 1972).

Podocyte damage. Extracapillary injuries manifest as podocyte damage. They have a very limited potential for repair. There is accumulating evidence that podocytes are unable to replicate postnatally as suggested by the lack of an increase in podocyte cell number during both postnatal and compensatory growth (Fries et al. 1989, Nagata et al. 1992 & 1993, Pabst et al. 1983). Exceptions to this rule are collapsing idiopathic glomerulosclerosis and HIV-associated nephropathy, where podocyte proliferation occurs (Barisoni et al. 1999). Podocytes support and maintain the integrity of the glomerular filtration barrier. Mutations in molecules that have important roles in podocytes, including nephrin, podocin, CD2AP, and α-actinin-4, result in proteinuria and glomerular sclerosis (Kestilä et al. 1998, Shih et al. 1999, Boute et al. 2000, Kaplan et al. 2000, Pollak 2002, Kim et al. 2003, Kos et al. 2003, Roselli et al. 2004). Experimental studies, particularly in the rat model of PAN (Kim et al. 2001), suggested a role of podocyte injury and depletion in the development of glomerular sclerosis and disease progression. In patients with types 1 and 2 diabetic glomerulosclerosis or IgA nephropathy, the number of podocytes was reduced in proportion to the severity of injury and degree of proteinuria (White et al. 2002, Pagtalunan et al. 1997, Lemley et al. 2002). These findings indicate that podocyte injury is a key initial step that triggers the pathologic sequence of events that leads to glomerulosclerosis (Pollak 2002, Kerjaschki et al. 2001, Mundel et al. 2002, Pavenstadt et al. 2003).

Direct evidence that podocyte loss is sufficient to induce glomerulosclerosis came only recently from studies with genetically engineered rodents. These experiments helped to define a causal relationship between podocyte damage and glomerulosclerosis. Asano et al. (2005), Matsusaka et al. (2005) and Wharram et al. (2005) designed transgenic rodent lines where selective podocyte injury could be induced. The findings were essentially the same in all three models of targeted podocyte damage. Most importantly, podocyte damage caused proteinuria and lead to glomerular sclerosis. In mice, podocyte changes included foot process effacement, detachment, and downregulation of SD proteins (Matsusaka et al. 2005). Endothelial cell swelling, mesangial cell proliferation, matrix expansion, and mesangiolysis, as well as damage and proliferation of parietal epithelial cells (PEC) followed. Interestingly, mesangial changes appeared after the podocyte injury but before podocytes were lost (Matsusaka et al. 2005). The degree of podocyte loss could be controlled and based on that Wharram and co-workers (2005) defined three stages of glomerular damage in rats. Stage 1, depletion of less than 20% of podocytes, caused mesangial expansion and transient proteinuria; stage 2, 20–40% depletion, led to focal segmental glomerulosclerosis and mild persistent proteinuria but normal renal function; and stage 3, loss of more than 40% of podocytes, resulted in segmental to global glomerulosclerosis, sustained high-grade proteinuria, and decreased renal function.

Since segmental scarring of glomeruli can be seen in many glomerular diseases at some point during the progression of the disease, it was initially postulated that the pathogenesis of FSGS follow one common pathway. Numerous studies in various rat models of FSGS have examined the pathogenesis of FSGS (Kretzler et al. 1994; Kriz et al. 1994, 1998a, 1995, 1996, & 2001; Nagata et al. 1992). These studies resulted in the theory of "podocyte depletion". It is characterized by the following sequence of events that ultimately lead to FSGS (Figure 5): (1) podocyte injury, resulting in podocyte loss with denudation of the GBM; (2) adherence of the PEC to the naked GBM; (3) formation of an adhesion between the GBM and Bowman's capsule; and (4) misdirected filtration of proteins into the periglomerular and peritubular space (Kriz et al. 1998a, 1998b, 1999a, & 1999b). These findings are supported by studies that show podocytes in the urine of patients and experimental animals with proteinuria (Petermann et al. 2003 & 2004, Vogelmann et al. 2003, Nakamura et al. 2000a & 2000b, Hara et al. 1995 & 2001). Currently, it is believed that after podocyte damage, an alternative mechanism leading to glomerulosclerosis is epithelial cell hyperplasia in glomeruli (Kriz and LeHir 2005). It is debated whether these epithelial cells are mainly of visceral or parietal origin, both in idiopathic forms of FSGS and in FSGS caused by HIV infection (Asano et al. 2005, Smeets et al. 2003; 2004, & 2006, Oyanagi et al. 2001, Moeller 2004). In addition to the detailed mechanisms, during the past years, proteinuria has been associated with a number of morphologic variants of glomerular sclerosis, both in human diseases and animal models (Fogo et al. 2003, D'Agati 2003). In some types of FSGS the early lesions in the glomerular tuft show predilection to perihilar or urinary pole (Le Hir et al. 2003, Thomas et al. 2006).

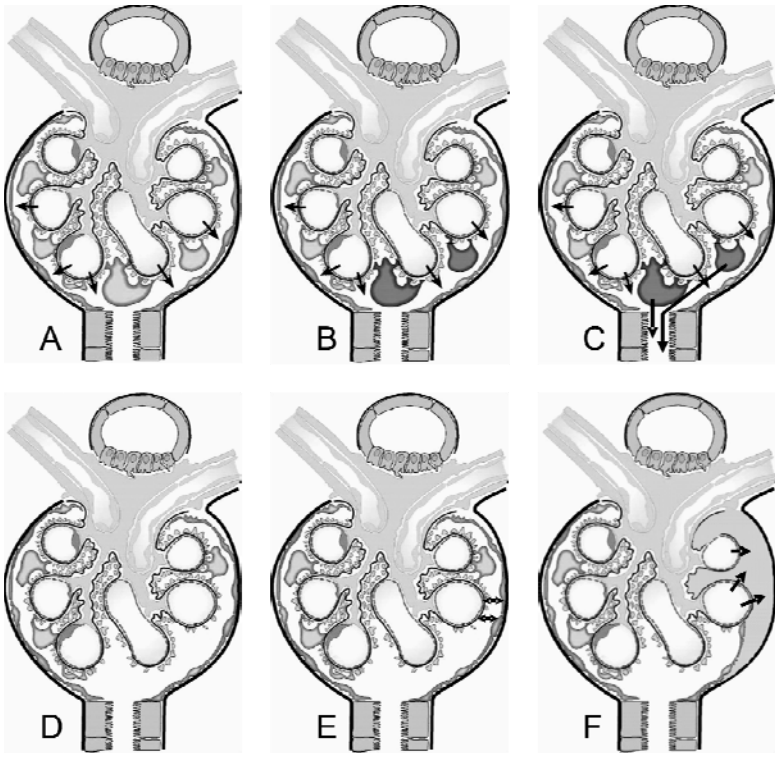


Figure 5 *Podocyte depletion theory. Sequence of events: (A) normal, (B) podocyte injury, (C) podocyte depletion, (D) bare areas of GBM in sites of podocyte depletion, (E) attachment of parietal epithelial cells to the naked GBM, (F) formation of a segmental lesion and misdirected filtration of urine. Modified from the original drawing by W. Kriz.*

2.3. Tubulointerstitial fibrosis

All chronic diseases causing progressive renal failure, whatever the initiating injury, are associated with tubulointerstitial changes including tubular degeneration and interstitial cell infiltration and fibrosis (Nath 1992). The severity of chronic tubulointerstitial lesions is the single best histologic correlate of the decline in renal function and longterm prognosis (Risdon et al. 1968, Schainuck et al. 1970, Mackensen-Haen et al. 1992, Kim et al 1995). In most kidneys (from patients) with progressive renal failure, the interstitium is expanded by a varying combination of events. These include widened tubular cell basement membranes (the main collagenous constituent of which is collagen IV), infiltration with mononuclear leukocytes and cells of the fibroblastic lineage, and gradual replacement of the interstitial areas with fibrous tissue (mainly collagen I, III and IV) (Norman and Fine 1999), associated with local tubular degeneration and dilation. An integral feature of interstitial fibrosis is its effect on tubulointerstitial cells: the tubules and peritubular capillaries ultimately disappear (Eddy 1996, Becker and Hewitson 2000).

Role of TECs. The tubules are uniquely challenged in high-grade proteinuria (Burton and Harris 1996). Filtered albumin and other proteins that accumulate within intracellular compartments of proximal TECs may perturb cell function by several mechanisms

(Remuzzi and Bertani 1998). Details of specific mechanisms linking the excess traffic of plasma proteins and interstitial injury have been derived from in vitro studies using polarized proximal TECs as a model to assess the effects of apical exposure to proteins. By addition of albumin, immunoglobulin G (IgG), or transferrin, the rate of synthesis of endothelin-1 (ET-1) in proximal TECs was enhanced several times in a concentration-dependent fashion (Zoja et al. 1995). Similarly, albumin and transferrin have been shown to upregulate monocyte chemoattractant protein (MCP-1) gene (Wang et al. 1997). Various investigators have reported stimulatory effects of a diversity of plasma proteins on the expression of proinflammatory and profibrotic mediators in renal TECs (Ruggenenti and Remuzzi 2000). The mediators include RANTES (Regulated upon Activation, Normal T Cell Expressed and Secreted) (Zoja et al. 1998), MCP-1 (Bohrer et al. 1977, Zoja et al. 1995), interleukin 8 (IL-8) (Mathiesen et al. 1991), fractalkine (Wang et al. 1997). TECs synthesize these mediators also in vivo and for example tubular MCP-1 production has been documented both in human diseases (Grandaliano et al. 1996) and in animal models (of chronic renal disease) (Tang et al. 1997, Tesch et al. 1999b, Okada et al. 2000). In response to protein load, TECs also synthesize specific complement components, some of which are pro-inflammatory, including C3 and C4 (Tang et al. 1999).

Intracellularly, protein overload has been shown to lead to protein kinase C (PKC) - dependent oxygen radical generation, subsequent nuclear translocation of transcriptional factor NF-κB and consequent gene upregulation in proximal TECs (Zoja et al. 1998, Wang et al. 1999b, Drumm et al. 2003, Tang et al. 2003, Morigi et al. 2002). Inhibition of this pathway with specific PKC inhibitors prevented MCP-1 and IL-8 upregulation (Tang et al. 2003). Later studies showed that proteinuria also activated other signaling pathways leading to cytokine production such as mitogen-activated kinase p38, extracellular signal-regulated kinase 1 and 2 (ERK1/ERK2), and the signal transducer and activator of transcription (STAT) proteins (Donadelli et al. 2003, Dixon et al. 2000, Nakajima et al. 2004).

The reason, why the synthesis of these proinflammatory mediators by TECs is so interesting, is that progressive renal disease is characterized by an interstitial infiltrate of mononuclear cells. Monocytes and macrophages in particular are thought to directly contribute to the fibrogenic process as a source of several profibrotic molecules (Nathan 1987). This has been established in several experiments, that have reduced the number of interstitial macrophages by immunosuppressive therapy, or by inactivation of intrarenal mediators of monocyte recruitment such as MCP-1 and RANTES (Gattone et al. 1995, Romero et al. 1999, Tesch et al. 1999a & 1999b, Shimizu et al. 2003, Anders et al. 2002, Eis et al. 2004, Kitagawa et al. 2004). Macrophages may synthesize several molecules that contribute to the fibrogenic process, perhaps the most important of which are the profibrotic growth factors, transforming growth factor-beta (TGF-β) in particular. Based on current evidence, TGF-β is the grand master of renal fibrosis. This has been proved by several ways of either inactivation or activation of TGF-β in experimental animal models (Ziyadeh et al 2000, Fukasawa et al. 2004, Zhang et al. 2003, Sato et al. 2003, Mozes et al. 1999, Lan et al. 2003).

Irrespective of the initial causes, interstitial fibrosis is a remarkably monotonous process characterized by de novo activation of α-smooth muscle actin (α-SMA) positive

myofibroblasts, the principal effector cells that are responsible for the excess deposition of interstitial extracellular matrix (ECM) under pathologic conditions (Roberts et al. 1997, Powell et al. 1999, Badid et al. 2002). The normal interstitium is populated with a small number of fibroblasts that are relatively quiescent, but they may proliferate and undergo phenotypic transformation to α-SMA positive myofibroblasts under certain condi-tions. Such α-SMA expressing cells, restricted to periarteriolar regions in normal kidneys, densely populate the interstitium of chronically damaged kidneys (Eddy 2005). Platelet-derived growth factor (PDGF) and TGF-β are two growth factors that promote fibroblast proliferation and TGF-β also transformation into myofibroblasts (Tang et al. 1996, Zeisberg et al. 2003). A subset of the myofibroblasts may also be bone marrow -derived (Eddy 2005).

The tubules may also be directly involved in the fibrogenic process. Profibrotic growth factors such as insulin-like growth factor-1 (IGF-1) and TGF-β can filter into the urine during proteinuria, bind to their cognate receptors on the apical surface of TECs and stimulate the synthesis of matrix proteins (Wang et al. 1999a). It has also been suggested that TECs are a significant source of interstitial myofibroblasts in chronic renal disease. These cells have been shown to transdifferentiate into myofibroblasts through epithelial-mesenchymal transition (EMT). In this process, the signals produced locally within damaged kidneys, most importantly TGF-β, engage transition of epithelial cells to mesenchymal cells that lose epithelial proteins, begin to express mesenchymal proteins such as vimentin and α-SMA, and gain access to interstitial spaces after the disruption of tubular basement membranes by locally produced proteases such as some of the metalloproteinases (Kalluri and Nelson 2003, Liu 2004a). The importance of the EMT process in renal fibrosis has been highlighted by studies of antifibrotic molecules hepatocyte growth factor (HGF) and bone morphogenic protein-7 (BMP-7). Several studies have showed their renoprotective effects and they both prevent EMT (Zeisberg et al. 2003 & 2005, Liu 2004b, Yang and Liu 2003, Gong et al. 2004, Zhang et al. 2005).

Aims of the study

The Finnish NPHS1 children form a genetically homogenous group of patients with a constant heavy proteinuria. Their nephrectomized kidneys offered a unique possibility to study the pathophysiology of this process. The specific aims of this study were to investigate:

- the development of glomerular and tubulointerstitial lesions in human kidneys with heavy proteinuria. (I, II)

- the expression of nephrin in extrarenal tissues in man and the functional significance of nephrin deficiency in specified organs. (III)

- the pathomechanisms and mangement of post-transplantation nephrotic syndrome in kidney grafts of NPHS1 patients. (IV)

Materials and methods

1. Tissue, serum and urine samples (I-IV)

A total of 49 kidneys were removed from children with NPHS1 between 1986 and 2003 (I-II). Eight adult kidneys unsuitable for TX were used as controls (I-II). Porcine kidney samples were obtained from two newborns and one 6-month-old pig (II, III). For the study of nephrin expression (III), human fetal samples were collected at autopsy from three fetuses aborted due to trisomy 21. Tissue samples were also obtained at autopsies of a preterm baby and four infants. Two human testis samples came from orchiectomies of adult men. Porcine fetal samples were obtained from aborted pregnancies. The NPHS1 samples were obtained from two infants who had died after renal TX at the ages of 2 and 3 years. For the search of podocytes in the urine (I), fresh urine samples were collected from four NPHS1 children, five pediatric patients with normal kidney function, and five healthy adults. Serum samples from 11 NPHS1 children with recurrent NS were collected for analysis of anti-nephrin antibodies (IV). As controls we used serum samples from 89 children with liver and heart TX as well as non-NPHS1 children with kidney TX. Percutaneous core needle biopsies taken at the onset of recurrent nephrosis from 12 NPHS1 patients were analyzed for immunological attack (IV). Routine biopsy samples taken from 20 non-NPHS1 children with a transplant were used as controls (IV).

2. Collection of clinical data (III-IV)

For the study of nephrin expression, clinical records of 56 NPHS1 children were analyzed retrospectively, and the data regarding the neurological findings and pancreatic function were recorded (III). To study the function of pancreatic beta-cells, an oral glucose tolerance test was performed on 36 NPHS1 patients and 25 other kidney transplant patients 1 to 5 years after renal TX. Serum sex hormone levels were measured from eight 15 to 17 year-old boys with NPHS1 to study testicular function (III). The control group consisted of 10 age-matched boys who had received a kidney transplant for other reasons. To study the effect of plasma exchange (PE) on recurrent nephrosis (IV), clinical data on 65 NPHS1 patients with renal TX were collected. The operations were performed at the Hospital for Children and Adolescents, University of Helsinki between the years 1986-2006. The data on the GFR, biopsies and clinical features of nephrotic episodes were retrospectively analyzed and the outcome after recurrence was recorded.

3. Antibodies (I-IV)

Rabbit polyclonal antiserum directed against the intracellular part of nephrin molecule was used for the immunohistochemistry of nephrin (III). This antiserum was prepared as described previously (Ruotsalainen et al. 1999). Several commercial antibodies were also used as described in detail in the original publications (I-IV).

4. Immunofluorescence staining (I-IV)

Cryosections (5 μm) of the tissue samples were fixed with 3.5% paraformaldehyde or acetone, depending on the antibody used. The sections were incubated overnight at 4°C with antibodies diluted in phosphate buffered saline (PBS). Fluorophore-conjugated secondary antibodies were incubated for 1 h. Each incubation was followed by three 5-min washes with PBS. Sections used for control were incubated in PBS instead of a primary antibody. The urine sediments of NPHS1 patients and controls (I) were studied by immunofluorescence as described previously (Hara et al. 1995).

5. Immunoperoxidase staining (I, II, IV)

For immunoperoxidase staining, sections of formalin-fixed, paraffin-embedded tissue samples were deparaffinised and endogenous peroxidase activity was quenched. To improve antibody penetration, microwave treatment with 10mM citric acid or commercial solution was used depending on the antibody. Amplification of the primary antibody reaction was achieved by incubating the sections with biotinylated secondary antibody. The binding was visualized using chromogene, and the sections were counterstained with hematoxylin. Immunoperoxidase staining of cryosections, fixed with 3.5% paraformaldehyde or acetone, was performed similarly.

6. Immunoblotting (II-III)

For the western blotting, tissue samples were homogenized with a dispenser in Laemmli sample buffer, protein concentration was measured using a modified Lowry assay, and the proteins were separated on a gel (7%-12%) and blotted onto an polyvinylidene fluoride membrane (PVDF). After blocking with 5% nonfat dry milk in PBS, the membrane was stained with a primary antibody followed by a peroxidase-conjugated secondary antibody. Bound antibodies were visualized using enhanced chemiluminescence.

7. Enzyme-Linked Immunosorbent Assay (ELISA) (IV)

Specific anti-nephrin antibodies were measured by ELISA using microtiter plates coated with the extracellular part of nephrin. The plates were incubated in blocking solution, followed by serum samples, and then by peroxidase conjugated anti-human IgG. Peroxidase substrate 1,2-phenylenediamine dihydrochloride was added after washes. When the color had developed the absorbance was read at 490 nm. Threshold for anti-nephrin antibody positivity was set to an absorbance representing the 95th percentile of the controls. The binding was specific for nephrin as shown earlier by a competitive assay (Patrakka et al. 2002).

8. Cytokine array (II)

The antibody array consisted of 50 different human cytokine and chemokine antibodies spotted in duplicate onto a membrane. The tissue samples of kidney cortex were homogenized in lysis buffer and centrifuged. Protein concentration was measured using a modified Lowry assay. The membranes were incubated with a blocking buffer and the tissue lysate was added to the membrane and incubated over night at 4◦C. After washes, the membranes were incubated with biotinylated antibody for 2 hours, washed, and incubated for 1 hour with horse radish peroxidase (HRP) -conjugated streptavidin. The membranes were developed with the chemiluminescence system. Signal intensity was quantified with a freeware image analysis software. All values were compared with an internal positive control.

9. Light microscopy (I, II, IV)

Light microscopy was performed with a standard light microscope equipped with a digital camera. The histological lesions in the NPHS1 kidneys were evaluated by light microscopy using paraffin-embedded tissue sections stained with hematoxylin and eosin (HE) or periodic acid silver methenamin (PASM). Glomerular sclerosis (I) was correlated to the age at nephrectomy and the progression of glomerular damage was analyzed by grading ten features of glomerular histology. Long-term allograft nephropathy after recurrent nephrosis (IV) was analyzed from biopsies taken after recurrence and from routine biopsies of NPHS1 grafts with no recurrence. The scoring was based on Banff classification and the “chronic allograft damage index” (CADI) (Racusen et al. 1999, Isoniemi et al. 1992, Qvist et al. 1998, Qvist et al. 2000).

To calculate the area fraction of a particular immunostained component (I, II) images were imported to an Image J freeware image analysis program. Sequential grayscale images were grabbed and a threshold was applied to each image at a constant level that distinguished between the stained component and the background. The proportion of black to white pixels in the image was then calculated as a percentage (Furness 1997). Similarly,

the capillary lumen area (I, II) was calculated by colouring the lumens inside the CD34 stained endothelium and then setting the threshold for the applied colour.

10. Scanning electron microscopy (I)

The glomeruli for scanning electron microscopy (SEM) were isolated under dissection microscope immediately after a biopsy was taken. The samples were processed as previously described (Lahdenkari et al. 2004). In brief, the samples were fixed with phosphate buffered glutaraldehyde or osmiun tetroxide, washed, dehydrated, mounted under a microscope on aluminum stubs and finally sputter coated with platinum or chromium.

11. *In situ* hybridization (III)

In search for nephrin mRNA, formalin-fixed, paraffin-embedded sections (10 μm) were deparaffinized, rehydrated, and treated with proteinase-K, and subjected to *in situ* hybridization, as described previously (Wilkinson 1992). Briefly, the tissue sections were washed, acetylated and dehydrated, and then incubated with 33P-labeled antisense and sense riboprobes. After washes, RNA digestion, and dehydration, the sections were dipped in NTB2 nuclear emulsion and exposed in the dark for 1 or 2 weeks. After developing, the sections were counterstained with HE. The probes for *in situ* studies were synthesized by subcloning a 287-bp cDNA fragment corresponding to exon 10 in human *NPHS1* into pBluescript, and the antisense and sense RNAs were produced using T3 and T7 RNA polymerases, respectively (Kestilä et al. 1998).

12. Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) (I, II)

The occurrence of apoptosis in renal cells was analyzed by monitoring the presence of DNA fragmentation (Tilly 1994). Briefly, the slides were analyzed by light microscopy after counterstaining with hematoxylin. Cells exhibiting dark brown staining from the colorimetric reaction were considered positive for DNA fragmentation. Negative controls, conducted by omitting the labeling enzyme, yielded no reaction product. As positive controls, testis samples with abundant apoptosis were used.

13. Glutathione analysis (II)

The oxidative stress in the kidney cortex samples was evaluated by measuring the total and free glutathione levels (Ahola et al 1999). In brief, free sulfhydryls of thiols were derivatized with monobromobimane to form fluorescent complexes. Thiols were then separated with high-performance liquid chromatography (HPLC) and detected fluorometrically to find out the concentration of free thiols. In order to measure the total (reduced + oxidized) concentration of glutathione, the samples were first treated with dithiothreitol to reduce disulphides.

14. Statistics

Unpaired Student's t-test was used in the analysis of the glomerular area (I), the oxidative stress of NPHS1 kidneys vs. controls in glutathione analysis (II), the GFR of patients with and without recurrence (IV), and the differences in the expression of various proteins examined by immunohistochemistry or Western blotting(I, II, IV). Correlation coefficient was calculated for analysis of linear correlation between histological findings of NPHS1 glomeruli (I) and development of renal lesions over time (I, II).

15. Ethics

The sample collection and procedures described herein were approved by the Ethics committees' of the Hospital for Children and Adolescents (I-IV) and the Department of Obstetrics and Gynecology (III), University of Helsinki and conformed to the principles outlined in the Declaration of Helsinki.

Results

1. General histology of NPHS1 kidneys (I, II)

In light microscopy of 49 NPHS1 kidneys nephrectomized at the age of 4 to 44 months the histology ranged from nearly normal to severely damaged. The findings included glomerular sclerosis, interstitial fibrosis and inflammation, arterial wall thickening, tubular atrophy and dilatation, and accumulation of proteinaceous material into tubular lumen. Fairly normal and totally sclerotic glomeruli and tubuli could be seen next to each other. Affected glomeruli were scattered around the whole cortex, without predilection to juxtamedullary area as often seen in FSGS. Interstitial fibrosis was first evident in the vicinity of glomeruli and sclerotic arteries as well as around degenerating tubuli. Due to fibrosis the peritubular space became progressively wider.

Immunoperoxidase staining and Western blotting showed increased amounts of collagen type I and type VI, vimentin, and a-SMA in the interstitium of the NPHS1 kidneys as compared to controls. Based on the histological scoring, there was a considerable variation in the rate of progression of the renal lesions. Nevertheless, a weak positive correlation (r^2=0.34) to the age of the patient at nephrectomy was found.

2. Glomerular lesions in NPHS1 kidneys (I)

Systematic histological analysis of over 1500 glomeruli of 20 NPHS1 kidneys showed that mesangial sclerosis was an early and prominent event. Mesangial changes from mild widening of the mesangial stalk to total sclerosis were seen in 99.7% of the glomeruli. The early lesions were scattered around the glomerular tuft and consisted of increased number of mesangial cells and matrix (Figure 6). The number of proliferating cells in the glomerular tuft, as revealed by the mindbomb homolog 1 (MIB-1) staining, was significantly increased in the NPHS1 as compared to controls (1.1 vs. 0.2 per glomerular cross section [GCS]). There was very little apoptosis with no difference between NPHS1 and controls (0.01 vs. 0.07 per GCS) in TUNEL.

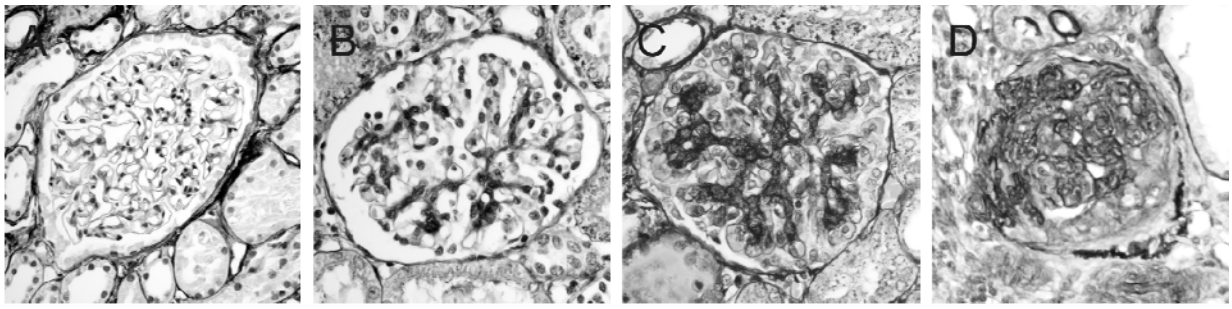


Figure 6 *Glomerular sclerosis in NPHS1 kidneys. (A) Normal glomerulus. (B) NPHS1 glomerulus with nearly normal histology except for the minor mesangial accumulation of connective tissue. (C) Characteristic lesions in an NPHS1 glomeruli. Increase in mesangial cells and matrix (arrows) without signs of crescent formation. (D) Typical view of a totally sclerosed NPHS1 glomerulus. Periodic acid silver methenamin (PASM) stainings.*

The podocyte structure was altered in NPHS1 glomeruli as shown by electron microscopy. SEM revealed prominent podocyte cell bodies with microvillous degeneration, elongated primary processes, and foot process effacement. Long primary processes and prominence of the cell bodies were also seen in light microscopy. Immunohistochemistry revealed that (1) the podocytes in NPHS1 glomeruli were in a hypertrophic state; (2) there were very few proliferating podocytes (0.08 MIB-1 positive cells/GCS), (3) there was no podocyte layer hyperplasia, (4) the number of Wilms tumor 1 (WT1) cells (podocytes) in well preserved glomeruli was normal (33/GCS).

We found very few TUNEL positive podocytes or podocytes with condensed nuclei, and the expression of pro-apoptotic p53, and anti-apoptotic B-cell lymphoma 2 (Bcl-2) proteins did not significantly differ from controls. The expression of podocyte markers (WT1 and vimentin), however, was decreased indicating podocyte loss in clearly sclerotic glomeruli. This raised a question of podocyte detachment, which was evaluated by counting WT1-positive cells in tubular lumens of formalin-fixed tissue samples and by double immunostaining the cells in NPHS1 and control urine samples with podocyte markers podocalyxin and zonula occludens-1 (ZO-1). No WT1 positive cells were found inside tubuli, but a median of 13.4 co-positive cells/ml was found in NPHS1 urine samples as compared to 0.3 cells/ml in controls.

Interestingly, PECs lining the Bowman's capsule were resistant to the protein overload in the primary urine since there was no difference in the rate of apoptosis or proliferation of these cells in NPHS1 kidneys and controls. PEC layer looked normal in 91% of the glomeruli and more than one layer of PECs was seen in only one glomerulus out of over a thousand. However, hypertrophic single-cell layer was observed in 9% of the glomeruli, mostly (87%) in association with thickened basement membrane. Contact of the capillary tuft with the Bowman's capsule was quite common both in NPHS1 and control kidneys. However, segmental crescent was seen in only 0.5% of the glomeruli analyzed and protrusion of the capillary tuft through Bowman's capsule was not seen. Lesions at the tubular opening were very rare. Widening of the urinary space was seen in 18% of the NPHS1 glomeruli and was due to shrinkage of the glomerular tuft.

3. Tubular epithelium in NPHS1 kidneys (II)

In light microscopy, proximal TECs contained protein and lipid rich vesicles and dilatation of tubules with flattening of epithelial cells was quite common. Proliferation or apoptosis of TECs in NPHS1 kidneys did not differ significantly from controls. The expression of TEC proteins megalin and cytokeratin were comparable in control and NPHS1 kidneys with normal histology. However, TECs positive for the mesangial marker vimentin were present in relatively high numbers, significantly more in NPHS1 than control kidneys. Nevertheless, no evidence of EMT was seen, as only four cells positive for α-SMA were found among approximately 117,000 TECs. Also, the TECs did not express type I collagen and immunofluorescence staining for matrix metalloproteinases 2 and 9 (MMP-2 and -9) revealed very few positive TECs. Furthermore, tubular basement membrane ruptures were not seen more often than in controls in light microscopy. Of note is that sharp borders between the degenerating and healthy tubular profiles were typically seen.

4. Inflammatory cells in NPHS1 kidneys (I, II)

Clusters of inflammatory cells first appeared in the vicinity of degenerating glomeruli in NPHS1 kidneys. In the glomerular capillary tuft, inflammatory cells were rare as studied by immunohistochemistry and were mainly lymphocytes and monocytes. The number of peritubular inflammatory cells was less pronounced, and it was variable in the fibrotic areas surrounding the degenerating tubuli. The interstitial inflammatory cells in the peritubular area were mainly monocytes in NPHS1 kidneys while T lymphocytes were the second largest group. Proximal and distal tubules of NPHS1 and control kidneys contained comparable numbers of mononuclear cells. Interestingly, there was no tubulitis.

5. Expression of cytokines and growth factors in NPHS1 kidneys (II)

Kidney cortex samples were analyzed with an antibody array of 50 cytokines, chemokines, and growth factors. The most prominent finding in NPHS1 kidneys was extremely high expression of neutrophil activating peptide 2 (NAP-2) (Figure 7). Also, the levels of two monocytic chemokines, MCP-1, and macrophage inhibiting factor (MIF) were clearly elevated. In addition, several other soluble mediators were up-regulated: angiogenin, intercellular adhesion molecule-1 (ICAM-1), interleukin receptor antagonist (IL-1ra), tumor necrosis factor-α (TNF- α), epidermal growth factor (EGF), and RANTES. In immunofluorescence staining, the monocytic chemokine MCP-1 and mono- and lymphocytic chemokine MIF were mostly detected in interstitial cells and less so in tubular epithelium. NAP-2 also showed staining in interstitial cells, but, in addition to this, part of the TECs expressed NAP-2 in NPHS1 kidneys. Surprisingly, the major profibrotic

factor TGF-β level was surprisingly low in the cytokine array and did not significantly differ in NPHS1 and control kidneys. The staining of TGF- β revealed no staining of tubuli and a scattered staining of interstitial cells in NPHS1 kidneys. NPHS1 kidneys contained significantly more VEGF in the cytokine array compared to controls. The pattern of immunofluorescence staining was similar to that in control kidneys.

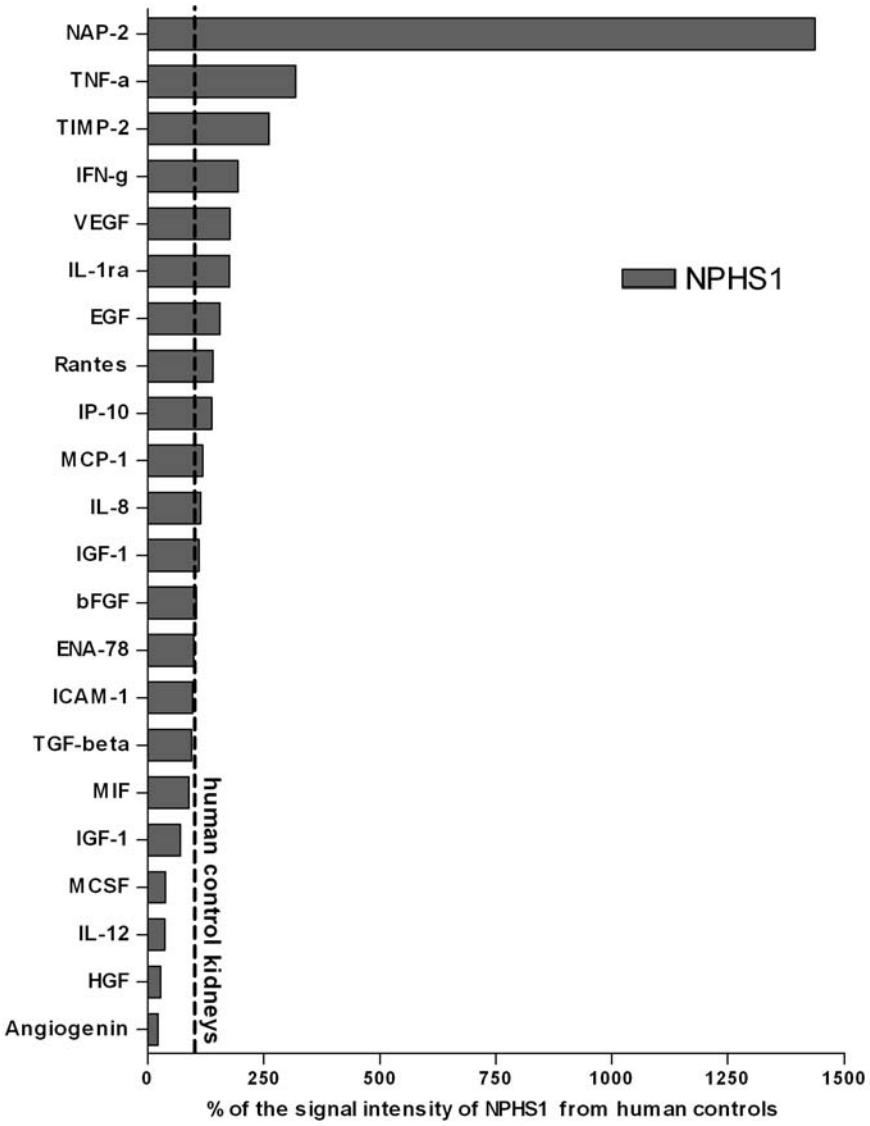


Figure 7 *Antibody array analysis of the cytokines of NPHS1 renal cortex. Relative signal intensity of the cytokines in NPHS1 kidneys shown as a percentage from the signal intensity of the respective cytokine in human control kidneys. The dottet line is at 100% and represents the level of the cytokines in human control kidney cortex. The 12 uppermost cytokines are more abundant in NPHS1 kidneys than in controls. An array of 50 cytokines was used and the cytokines with levels above the detection limit of the array are shown here.*

6. Glomerular and peritubular capillaries (I, II)

Decrease in the surface area of capillary lumens was seen both in the glomerular tuft and in the peritubular capillaries of the interstitium. Marked changes in the capillaries could already be seen in glomeruli with mild and moderate mesangial changes. Immunoperoxidase staining for the endothelial marker CD34 showed decrease in the total endothelial surface area and narrowing of the capillary lumens of glomeruli. Endothelial cell swelling was evident in light microscopy, but capillary thrombosis or hyaline occlusion was not seen. Collapse of the capillary tuft was evident in glomeruli with severe mesangial lesions. The peritubular capillary endothelial surface area was unaltered, but capillary lumen size was diminished by 47% (Figure 8).

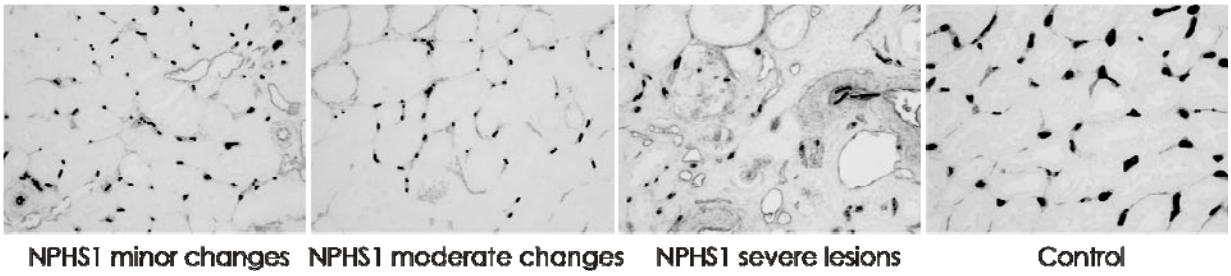


Figure 8 *Peritubular capillary lumens in different stages of renal damage in NPHS1 and control kidney interstitium. Capillary endothelial staining with CD34 antibody and lumens colorized with black by digital image processing. Note the clear difference in the lumen size of the peritubular capillaries between NPHS1 and control already in NPHS1 kidneys with fairly normal histology (first image from the left).*

7. Oxidative stress (II)

NPHS1 kidneys were under a considerable oxidative stress. The concentration of free glutathione, representing the total oxidative stress in the tissue, was extremely low in NPHS1 kidney cortex as compared to controls. This was supported by the finding of high levels of myeloperoxidase positivity in the interstitium of NPHS1 kidneys.

8. Expression of nephrin in nonrenal tissues (III)

The expression of nephrin in normal human and porcine tissues was studied by means of immunohistochemistry, western blotting and *in situ* hybridization. With the use of a rabbit polyclonal antiserum, which was raised against the intracellular part of human nephrin, we did not detect positive immunofluorescence staining in any other organ than the kidney. The antiserum reacted strongly against the human and porcine glomeruli with a linear pattern of staining. A total of 31 fetal, newborn, and infant nonrenal tissue samples from human and pig were stained and all were negative. Similarly, Western blotting of 30

nonrenal tissues was negative. These results were supported by the *in situ* hybridization. The signal for mRNA corresponding the extracellular part of nephrin was strong in kidney glomerulus, but no signal above background was seen in the other 21 tissue samples studied. *In situ* hybridization was also performed to selected tissues of two NPHS1 patients (while they do not express nephrin, they do synthesize nephrin mRNA). The signal in glomerulus was strong while other tissues remained negative.

Most importantly, however, the phenotype was limited to the renal malfunction in majority of NPHS1 patients. Over 90% of the patients did not have motor or sensory dysfunction. None had diabetes and oral glucose tolerance test results for insulin and glucose were similar in 36 NPHS1 and 25 other transplant patients. 88% of the tested late- and post-pubertal NPHS1 boys had normal gonadal development in clinical examination and 75% had normal levels of gonadotropins, testosterone, and inhibin B.

9. Recurrent nephrotic syndrome in NPHS1 children (IV)

Twenty-three episodes of recurrent nephrosis occurred in 13 of the 65 children with NPHS1 transplanted in Hospital for Children and Adolescents between the years 1986 and 2006. All patients were homozygotes for the Fin-major mutation. 73% of these patients had anti-nephrin antibodies. Only minor glomerular lesions were observed in biopsies that were taken at the beginning of the recurrent nephrosis (Table 2). Of the 23 episodes, 20 were treated similarly by changing normal triple medication of cyclosporine (CsA), azathioprine (AZA) and methylprednisolone (MP) to triple therapy with CsA, cyclophosphamide (CP) and increased dose of MP. Since the year 2000, PE was used in addition to this medication. Eleven episodes were treated without and nine with PE. PE improved remission rate from 55% to 89%. PE therapy was given for 9 weeks on average with a mean of 26 exchanges.

Table 2. *Immunohistochemistry of markers for humoral and cell mediated inflammation in NPHS1 glomeruli during recurrent nephrosis.*

Antigen	NPHS1	Controls	*P*
	Positive biopsy samples (%)[1]		
IgA	20%	-	-
IgM	50%	-	-
IgG	30%	-	-
C1q	20%	-	-
C3	20%	-	-
	Positive focuses per GCS[2]		
C4d	0	0	-
MPO	0.54±0.26	0.31±0.32	NS
CD3	0.29±0.38	0.78±0.63	NS
CD20	0	0.05±0.07	NS

GCS; glomerular cross section, MPO; granulocytes (myeloperoxidase), CD3; B-lymphocytes, CD20; T-lymphocytes, NS; not significant

[1] Routine immunofluorescence results were available from 10 biopsies.

[2] Data from immunoperoxidase staining of 17 NPHS1 and 20 control biopsies.

Nine of the 23 episodes resulted in graft loss. All the patients who had lost a graft due to recurrent NS developed recurrence in the new graft within 1-22 days after the re-TX. In two patients, the first PE was performed preoperatively but it did not prevent the development of NS. The treatment of these patients with CP and PE, however, was successful in three of the four transplantations. Before the introduction of PE, two grafts were lost after the re-TX.

If remission was achieved, the episode did not have a strong impact on the renal function. There was no difference in creatinine values just before and three months after the episode (Figure 9). Also the GFR was comparable during a six-year follow-up in patients with and without recurrent NS. In addition, four years after the episode, the histology did not differ significantly from that in other NPHS1 transplants when analyzed with Banff chronic, Banff acute, and CADI classifications.

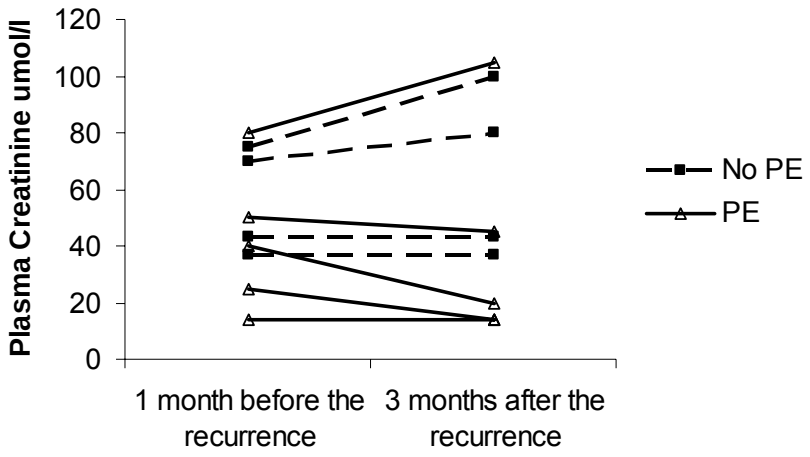


Figure 9 *Plasma creatinine concentrations before and after an episode of recurrent nephrosis in nine NPHS1 patients. There was no impairment in the renal function three months after the recurrence either in patients treated with plasma exchange (PE) or in patients treated without PE.*

Discussion

Understanding of the pathophysiology of proteinuria is important in nephrology. Proteinuria is not only a sign of a kidney disease but also a major factor leading to renal failure (Jafar et al. 2001, Ruggenenti et al. 1998, Peterson et al. 1995, De Zeeuw et al. 2004). Identification of the genetic basis of NPHS1 in 1998 (Kestilä et al.) and finding of nephrin as a major component of the podocyte SD (Ruotsalainen et al. 1999) started a new era in understanding the molecular basis of the glomerular filtration barrier. NPHS1 is a prototype of nephrotic diseases and these kidneys serve as a unique human model to study the effects of chronic proteinuria and podocyte damage to renal physiology and to study the events leading to renal damage. In NPHS1, the molecular defect is known, it affects equally all podocytes and glomeruli, and sufficient amount of kidney tissue is available for experimentation.

1. Pathology of NPHS1 kidneys (I-II)

1.1. Podocyte changes (I)

The primary lesion in NPHS1 glomeruli is located in podocytes which are unable to produce functional nephrin. Podocytes in NPHS1 undergo phenotypic changes, such as foot process effacement - a typical feature for nephrotic diseases (Fogo 2003). In the present study, they also showed hypertrophy, which was evident both in electron and light microscopy and was further supported by the findings of the upregulation of cell cycle promoters cyclin A and D1, and downregulation of cell cycle kinase inhibitor p57. As an isolated observation these changes in cell cycle regulatory proteins are a sign of hypertrophic and hyperplastic phenotype (Marshall and Shankland 2006). However, when found in parallel with low rate of proliferation it was clear that podocytes were in an activated, hypertrophic, state. The results support the idea that podocytes are terminally specialized cells which show proliferation only in very unique conditions such as in HIV nephropathy (Barisoni et al. 1999).

Detailed morphometrical analyses performed mainly by Kriz and co-workers in animal models of FSGS have indicated a sequence of events starting from podocyte depletion with denuded GBM and leading to formation of a segmental lesion and subsequent glomerular sclerosis (Kriz et al. 1998a & 2005, Gassler et al. 2001). This “podocyte depletion theory” has gained wide acceptance as the final common pathway of glomerular damage in proteinuria. Thus, an interesting question was whether the damaged podocytes in NPHS1 kidney would undergo apoptosis or detachment from the GBM as the prevailing theory suggests. Somewhat surprisingly we did not detect apoptotic podocytes and or evidence of the ensuing events described by Kriz and co-workers was observed. Shedding of podocytes into urine was observed in NPHS1, reflecting the podocyte injury and has been reported in several types of human kidney diseases (Vogelmann et al. 2003,

Nakamura et al. 2000a & 2000b, Hara et al. 1995 & 2001). The amount of urinary podocytes, however, was modest and most probably associated with the final glomerular destruction.

Attachment of the glomerular tuft with the Bowman's capsule was frequently seen both in NPHS1 and control kidneys in light microscopy. To gain better view of this phenomenon, electron microscopic images would have been helpful. However, if adhesion of the GBM to Bowman's capsule and subsequent crescent formation were an important early event leading to glomerular sclerosis and nephron degeneration, segmental lesions should have been present in a substantial proportion of the NPHS1 glomeruli analyzed. This was clearly not the case. Therefore we did not find electron microscopy necessary for confirmation of the true nature of GBM and Bowman's capsule contacts. The lack of actual adhesions between GBM and Bowman's capsule was also indirectly supported by transmission electron microscopy studies performed by Lahdenkari and co-workers (2004) that showed no bare areas of GBM in NPHS1 glomeruli.

1.2. Mesangial lesions (I)

Glomerular sclerosis in NPHS1 kidneys was found to develop through progressive mesangial sclerosis that led to obliteration of glomerular capillaries. Some degree of mesangial sclerosis was found practically in every glomerulus of NPHS1 kidneys. This is not a revolutionary concept of the pathogenesis of glomerular damage in NPHS1 kidneys, since Rapola and Vilska reported as early as 1972, that NPHS1 glomeruli showed mesangial cell proliferation with accumulation of a PAS- and silver- positive fibrillar matrix in the mesangium. In our systematic analysis of more than 1500 glomeruli, we did not find local protrusions of the glomerular tuft into the paratubular spaces or paraglomerular space, as reported in FSGS (Kriz et al. 1998a & 2005, Gassler et al. 2001). The early lesions in the glomerular tuft showed no predilection to perihilar or urinary pole and we did not find tip lesions or lesions in the urinary pole or perihilar areas. These lesions are all described in the literature in cases of some types of FSGS (Le Hir et al. 2003, Thomas et al. 2006, Fogo et al. 2003, D'Agati 2003). Immunohistochemistry revealed that the glomerular tuft contained low numbers of inflammatory cells, indicating that the increased cellularity of the endocapillary compartment was caused by hyperplasia of mesangial cells. Especially the low number of monocytes/macrophages was interesting, as these cells have been suggested to play a central role in crescent formation and glomerulosclerosis (Nikolic-Paterson et al. 1994, Hooke et al. 1987).

A direct damage to mesangium has not led to the development of total glomerulosclerosis in animal models (Ichikawa et al. 2005, Mauer et al. 1972). By contrast, a direct damage to podocytes has lead to irreversible changes and resulted in glomerular sclerosis (Ichikawa et al. 2005). The role of podocytes in glomerular sclerosis was further supported by the work of Kriz and co-workers that described a stepwise sequence of events starting from podocyte damage and leading to formation of a segmental scar (Kriz et al. 1998a & 2005, Gassler et al. 2001). Therefore, even though it has been known that podocyte damage also triggers mesangial cell activation,

endocapillary lesions have been considered self-limiting and reversible (Kriz et al. 1998a). In the light of our evidence this seems to be an oversimplified conception. In NPHS1 kidneys, podocyte damage did not lead to formation of segmental lesions, but induced a mesangial response that was powerful enough to totally destroy the glomerulus.

Some degree of mesangial sclerosis is a common finding in all known podocyte gene defects that lead to proteinuria and also in laminin beta 2 (LAMB2) mutations where proteinuria is caused by a defect in GBM structure (Zenker et al. 2004, Lenkkeri et al. 1999, Boute et al. 2000, Caridi et al. 2001, Michaud et al. 2003, Shih et al. 1999). Further studies are needed to clarify what the factors are that drive the mesangial cell response in NPHS1 and why an extracapillary damage in some experimental models and human diseases lead to glomerular sclerosis through FSGS rather than through "pure" mesangial sclerosis as observed in NPHS1.

1.3. Changes in the vasculature (I, II)

Blood vessels in NPHS1 kidneys were severely affected and alterations in arteries, arterioles, and capillaries were among the earliest abnormalities observed. Arterial and arteriolar wall thickening comprised of intimal sclerosis and accumulation of ECM around the vessels. The surface area of glomerular capillary endothelium and the area fraction of capillary lumens were markedly decreased in NPHS1. The luminal area was 35% smaller in NPHS1 than in controls. This means a decrease of over 50% in the conductance of the capillaries (the conductance of a vessel increases in proportion to the fourth power of the diameter).

In contrast to glomerular capillaries, the peritubular capillary endothelial surface area was unaltered. However, as in the glomeruli, the lumen size was significantly smaller in peritubular capillaries of NPHS1 than of controls. The vasculature of the kidney cortex has a unique architecture where peritubular capillaries receive their blood from the efferent arteriole of the "parent" glomerulus, i.e. the glomerulus of the same nephron (Figure 10). These results suggest that obstruction of the blood flow in the narrowed glomerular capillaries led to hypoperfusion of the peritubular capillaries which seemed to have a well preserved endothelium but a small diameter either because of lower blood pressure due to decreased blood flow or increased contractility of the capillaries. This could possibly lead to a chronic ischemia of the nephron. It is known that hypoxia regulates a wide array of genes, including many fibrogenic factors and could therefore be at least partly response for the interstitial fibrosis observed in NPHS1 kidneys (Norman and Fine 2006).

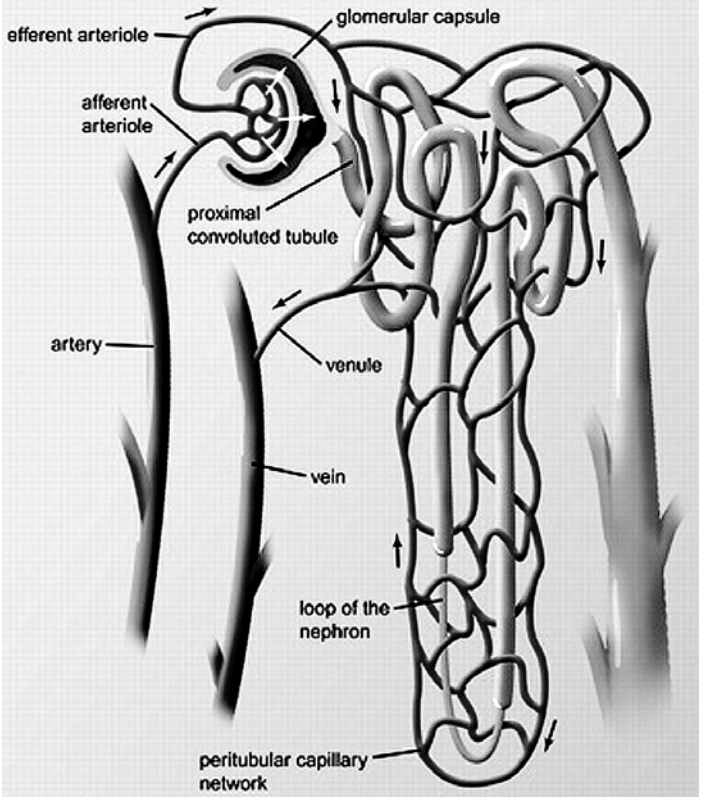


Figure 10 *The microvasculature of the nephron. The peritubular capillary plexus is fed by glomerular efferent arterioles and supplies nutrients and oxygen to tubular and interstitial cells. Adapted from Nangaku M: Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. J Am Soc Nephrol. 2006; 17(1):17-25. Used with permission of the copyright holder.*

1.4. Tubulointerstitial lesions (II)

Tubular epithelium in NPHS1 kidneys is faced with high intraluminal protein concentrations. Accordingly, accumulation of protein and lipid inside TECs, and flattening of the tubular epithelium and dilatation of the tubules were seen in NPHS1. Also, a varying degree of interstitial inflammation and fibrosis was manifest. It has been proposed, that TECs have a crucial role in progression of tubulointerstitial fibrosis and the following renal damage in diseases with chronic proteinuria (Harris and Neilson 2006). TECs have been reported to secrete profibrotic and proinflammatory cytokines and undergo EMT under proteinuria (Rastaldi 2006). TECs that have undergone EMT become myofibroblasts which are able to move from tubular epithelium into the interstitium and are very active producers of ECM. TECs are regarded as an important source of myofibroblasts by many authors, because the kidneys contain only a limited number of resident fibroblasts in the interstitium (Liu 2004a).

In NPHS1 kidneys, 10% of the TECs expressed intermediate filament protein vimentin (3% in control kidneys) and therefore had acquired mesenchymal properties. However,

TECs did not express smooth muscle actin (SMA), which is a hallmark of EMT. In addition, the expression of epithelial marker cytokeratin and protein of the apical endocytic apparatus, megalin, were normal in TECs of NPHS1 kidneys. TECs did not express matrix metalloproteinases (MMP) and tubular basement membranes had no more gaps than in controls. Practically no collagen type I expressing TECs were seen. Furthermore, the levels of the most potent EMT promoting factor, transforming growth factor β (TGF-β), were not higher in renal cortex of NPHS1 than of controls and TECs were negative for TGF- β in immuhistochemistry. Therefore, it is unlikely that EMT of TECs would play an important role in the production of interstitial fibrosis in NPSH1 kidneys.

TECs can participate in tubulointerstitial fibrosis also in indirect ways. Under proteinuric conditions, TECs are able to produce various chemokines that attract inflammatory cells into the interstitium and also produce profibrotic cytokines that increase the production of ECM in interstitial (myo)fibroblasts (Harris and Neilson 2006). We performed an antibody array analysis of over 50 cytokines from renal cortical samples. TECs constitute a great majority of renal cortical cells, and therefore renal cortex samples fairly accurately represent the situation of tubulointerstitium, not glomeruli. The most abundant chemokines in NPHS1 were NAP-2, MIF, and MCP-1. Interestingly, of these three, only NAP-2 was found to be partly produced by TECs in immunofluorescence whereas all three were expressed by solitary interstitial cells, most likely inflammatory cells. This is interesting, since MCP-1 is considered as one of the key cytokines produced by TECs with both chemotactic and profibrotic properties (Abbate et al. 2006). Of particular interest is also NAP-2. The level of this neutrophilic CXC chemokine (Brandt et al. 2000) was 16 times higher in NPHS1 than in control kidneys and it was expressed in the apical borders of TECs and solitary interstitial cells. To this date, very little is known about the role of NAP-2 in renal diseases, but it would certainly be an interesting target for further research.

The renal damage in NPHS1 kidneys was not transmitted through a local spreading in the renal interstitium, but was more likely due to events within individual nephrons. This was supported by the finding that well preserved areas of renal cortex were found next to severely damaged areas with clear-cut borders between them. If TEC reaction to excess protein in the urine was a key event in renal damage in NPHS1, one would have expected to find a more diffuse and universal interstitial fibrosis and inflammation. The fact that nephrons were destroyed relatively independently of the neighbouring nephrons, suggests the intranephronic events were crucial. Due to the unique vasculature of the kidney and the data presented in this study, an impairment of blood flow in the glomerular capillaries appears to be a major event in the pathogenesis of renal damage of NPHS1 kidneys.

An unexpectedly heavy oxidative stress was observed in renal cortex of NPHS1 patients. There were several possible sources of reactive oxygen species (ROS) in NPHS1 kidneys. High level of NAP-2 observed in the interstitium of NPSH1 kidneys may have been responsible for accumulation of neutrophilic granulocytes, as abundance of myeloperoxidase positive focuses were observed in the interstitium. A second possibility is that TECs produce ROS under proteinuric conditions (Eddy 2000). Third, the relationship between hypoxia and the production of ROS remains controversial. It has

however been shown in many studies that exposure of cells or tissues to moderate hypoxia (PO2 = 0.9–2.7 kPa) increases the production of mitochondrial-generated ROS (Chandel and Budinger 2007).

1.5. Synthesis of the histological studies (I,II)

Podocyte damage and hypertrophy triggered endocapillary activation in NPHS1. This manifested as proliferation of mesangial cells and increased production of ECM. Expansion the mesangium led to obliteration of the glomerular capillaries as both the surface area of the glomerular endothelium and the lumen size was decreased. The tubular epithelium was activated, as it gained some of the mesenchymal properties, and participated in the production of proinflammatory chemokines. However, the tubular epithelium seemed not to be the primary engine in driving interstitial fibrosis as it did not produce some of the main profibrotic cytokines and there was no EMT of TECs. A hypothetical explanation for interstitial damage in NPHS1 would be that this in turn, resulted in a significant decrease in the blood flow to the following capillary bed, namely peritubular capillaries of the same nephron. This was supported by the findings that the peritubular capillary endothelium was intact, but the lumen size was small. This possibly led to a chronic ischemia of the nephron. This hypothesis would explain the relatively independent progression of histological damage between adjacent nephrons but remains to be verified in future studies.

The research frame of the present study also poses some problems. The gene defect of NPSH1 is present from the first day of fetal growth, and it is possible that part of the abnormities observed in NPHS1 differ from those of kidneys that have initially been normal and are later in life faced with podocyte damage. Another hindrance of the studies with human material is the availability of control material. The human kidneys we used as controls came from adult cadaver donors. Their kidneys proved unsuitable for TX mainly because of vascular abnormalities. The kidneys already showed minor histological changes including slight accumulation of ECM in the interstitium. Brain death and a delay before freezing may also have an effect on the kidneys. Most likely, these factors play a role when tissue cytokine levels between NPHS1 and controls were compared. If we had had ideal controls, the sensitivity of cytokine testing would probably have increased.

2. The role of nephrin outside the kidney (III)

In the original study by Kestilä and co-workers (1998) nephrin was found to be podocyte specific. Later on, nephrin expression was reported in the brain, pancreas, and testis of mouse and in human pancreatic beta cells (Putaala et al. 2000 & 2001, Liu et al. 2001, Palmen et al. 2001). Since the knowledge of nephrin biology and possible manifestations of nephrin deficiency are important for the proper management of NPHS1 patients, we performed a survey of the nephrin protein and mRNA expressions using human and porcine tissues. No detectable level of nephrin protein was found in extrarenal tissues

with immunofluorescence and Western blotting. Also, *in situ* hybridization did not reveal nephrin mRNA in extrarenal tissues. After our study, nephrin expression has also been described in human pancreatic islet microendothelium (Zanone et al. 2005) and in lymphoid tissue (Åström et al. 2006). What the explanation for these different results is, is not known. The sensitivity of the methods or the specificity of the used anti-nephrin antibodies may have affected the results. In our work, the anti-nephrin antibodies detected the intracellular part of nephrin which has a unique structure. This is not the case with the extracellular part containing immunoglobulin and fibronectin domains (Kestilä et al. 1998).

A significant observation in our study was that the Finnish NPHS1 children, who completely lack nephrin, did not show defects in the pancreatic, testicular or brain function. Blood glucose and serum insulin levels after an oral glucose tolerance test in 36 NPHS1 patients were similar to those of 25 controls with a transplant. In addition, none of the NPHS1 patients had diabetes even though diabetogenic drugs, such as glucocorticoids and tacrolimus, were used after TX. None of the children had clinical symptoms that would indicate pathologic lesions in cerebellum, spinal cord, olfactory bulb, or the hippocampal area where nephrin has been found to be expressed in mice (Putaala et al. 2000 & 2001). No information on the fertility in NPHS1 was present because the oldest Finnish patients were in puberty. However, the pubertal development of male and female NPHS1 adolescents was clinically normal and the hormone synthesis of testicles was normal in six of the eight pubertal NPHS1 boys.

3. Recurrent nephrotic syndrome in kidney grafts of NPHS1 patients (IV)

Patrakka and co-workers described in 2002 episodes of recurrent NS in nine NPHS1 patients. All nine patients had a Fin-major/Fin-major genotype. Antibodies reacting against glomerulus in indirect immunofluorescence were found in eight, and high anti-nephrin antibody levels were detected with ELISA in four (44%) of the nine patients. Thus, circulating anti-nephrin anti-bodies seemed to have a pathogenic role in the development of heavy proteinuria in kidney grafts of NPHS1 patients. By the year 2006, at the end of our study, 13 patients had developed recurrent NS. Serum samples were available from 11 of these patients and we found specific anti-nephrin antibodies in eight (78%).

Interestingly, all 13 patients with recurrence had Fin-Major/Fin-major genotype and none of the samples taken before the first TX were positive indicating that the synthesis of the antibodies had started after the TX. Fin-major mutation leads to a peptide of only 90 amino acid residues (many of them being novel due to frame shift) and the complete absence of nephrin in the native kidney (Kestilä et al. 1998, Patrakka et al. 2000). Fetuses with this genotype do not encounter nephrin during normal maturation of their immunological system and do not develop tolerance to nephrin. When nephrin is exposed in the kidney graft after TX, immunization against this “novel” antigen may occur. This is analogous to that observed in Alport’s syndrome (Kashtan 1999).

There is no direct evidence of the disease causing ability of the anti-nephrin antibodies. However, a lot of indirect evidence has accumulated: (1) Anti-nephrin antibodies have been found in a great majority of NPHS1 patients with recurrent NS. (2) All patients with recurrence have been Fin-major homozygotes that are able to produce only a very short part of the nephrin protein. (3) CP that suppresses cell mediated humoral immune response has been effective in the treatment of recurrence. (4) PE that removes circulating factors from plasma effectively brought patients back to remission. (5) In patients with re-TX after graft loss due to a recurrent NS, heavy proteinuria developed within a few days after the new TX. This was indicative of an amplified attack against the glomerular filtration barrier and therefore conforms well to be a secondary immune response against a previously immunized antigen.

The immune reaction caused by the anti-nephrin antibodies was analyzed on a tissue level from kidney biopsies taken during recurrent NS. The biopsies showed only mild changes in light microscopy and no immunoglobulin or complement deposits. Also, no staining of C4d or B-cells was detected in glomeruli, which all speaks against an antibody mediated process. The paucity of findings may be explained by the data obtained from an animal model. In studies performed on rats by Orikasa and co-workers (1988), a single injection of mAb 5-1-6, which reacts with the extracellular part of nephrin, induced massive proteinuria, which started immediately and lasted for 18 days. No histological lesions were observed except for partial retraction of epithelial foot processes at the peak of proteinuria on day eight. Also, no IgG or C3 could be detected at any time during the course of the nephrosis (Orikasa et al 1988). On the other hand, immunoelectron microscopy showed that the mAb 5-1-6 antibody and nephrin underwent endocytosis into podocytes (Orikasa et al 1988). Later studies showed that quite a small amount of mAb 5-1-6 was capable of inducing proteinuria and also Fab fragments of mAb 5-1-6 were effective (Kawachi et al 1992, Narisawa et al. 1993). In addition, complement-depleted rats injected with mAb 5-1-6 IgG1 developed proteinuria with the same course as non-depleted rats (Narisawa et al. 1993). These observations and our findings in NPHS1 children suggest that anti-nephrin antibodies very effectively induce proteinuria even without the help of complement system or inflammatory cells. This may reflect the central role of nephrin for the podocyte SD and the glomerular filter.

Recurrent NS is common in kidney grafts of patients with focal FSGS. Mutations in *NPHS2* encoding for a podocyte protein, podocin, are a common cause of FSGS. It was initially assumed that the genetic forms of FSGS would not recur after TX. This, however, does not seem to be the case and recurrences have been reported also in patients with *NPHS2* mutations (Becker-Cohen et al. 2007, Carraro et al. 2002, Weber et al. 2004, Bertelli et al. 2003, Billing et al. 2004). Interestingly, anti-podocin antibodies have not been detected in the few patients studied so far by immunofluorescence or Western blot analysis (Becker-Cohen et al. 2007, Weber et al. 2004, Bertelli et al. 2003). Nephrin and podocin are both located in the podocyte SD area and interact with each other. An important difference between these molecules is that nephrin is a transmembrane protein with a large extracellular part while podocin is a cytosolic molecule in the podocyte foot process. The extracellular part of nephrin is most probably more immunogenic and more accessible to the antibodies than the intracellular podocin. This is supported by the studies

with mAb 5-1-6 antibodies and our preliminary findings, that the anti-nephrin anti-bodies react against epitopes located in the extracellular domains of nephrin (Kuusniemi et al., manuscript in preparation).

The recurrence in FSGS has been suggested to be caused by a "proteinuric factor" present in the plasma of these patients (Savin et al. 1996). This factor has been suspected also in patients with *NPHS2* mutations and post-TX proteinuria (Carraro et al. 2002). The factor has never been isolated, but its biological activity has been demonstrated by an in vitro permeability assay (Palb activity) (Savin et al. 1996). Interestingly, Srivastava et al. (2006) recently reported an NPHS1 patient, who developed recurrent NS after TX and showed increased Palb activity at the time of recurrence. No circulating anti-nephrin antibodies were detected and proteinuria resolved quickly after the commencement of CP treatment. The boy had remained in remission for four years after the TX in spite of the fact that the Palb activity remained elevated. Thus, it seems possible that different molecular mechanisms are involved in the development of the post-TX proteinuria. It must be emphasized, however, that the recurrence of NS in NPHS1 is very rare in other genotypes than the Fin-major/Fin-major. This mutation is a stop codon in the first exon of the NPHS1 gene, and for these children nephrin expressed in the kidney graft represents an immunologically foreign molecule. So, it seems probable that the "proteinuric factor" in this case is the anti-nephrin antibody.

Even without direct evidence, it seems very likely that circulating nephrin specific antibodies have a significant role in pathogenesis of recurrent NS. Worldwide, post-transplant recurrence of NS is a major problem in patients with FSGS. Despite extensive research, the etiology of the recurrence of FSGS and FSGS in general has remained unknown in the majority of individuals (Daskalakis and Winn 2006). The idea of antibodies against a podocyte protein is intriguing because diseases with podocyte gene defects and podocyte damage are very intensely studied at the moment. Furthermore, many new podocyte specific genes have been identified (Patrakka et al. 2007, Lehtonen et al. 2005) with possible involvement in the pathogenesis of FSGS and other renal diseases, and many more new proteins are on their way (He et al. 2007). It may be that specific anti-podocyte antibodies have a pathogenetic role in the recurrence of a subset of common renal diseases that are caused by a yet unknown gene defect of the podocyte.

Conclusions

The main results and conclusions of this study can be summarized as follows:

I. In the NPHS1 kidneys, podocyte damage and heavy proteinuria resulted in mesangial expansion and capillary obliteration which progressed to total glomerular sclerosis. Podocyte depletion, glomerular tuft adhesion to Bowman´s capsule, or misdirected filtration did not seem to be important in this process.

II. Glomerular protein leakage in NPHS1 kidneys resulted in interstitial fibrosis, inflammation, and oxidative stress as well as tubular dilatation and atrophy, which often was periglomerular and segmental at first. The tubular epithelial cells, however, did not show epithelial-mesenchymal transition.

III. The expression studies of nephrin protein or mRNA in human and pig tissues did not reveal significant quantities of nephrin anywhere else but in the kidney glomerulus. These findings were supported by the phenotype analysis of NPHS1 patients, who did not show major extrarenal signs or symptoms.

IV. The clinical and pathological data suggest that anti-nephrin antibodies effectively impaired the glomerular function in kidney grafts of NPHS1 patients homozygous for Fin-major mutation. Combination of plasma exchange and cyclophosphamide medication showed good efficacy in the treatment of post-transplant recurrence of proteinuria.

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Original publications