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The Role of Podocyte Genetics in Childhood Nephrotic Syndrome

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ACADEMIC DISSERTATION

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To my parents

“Ever tried. Ever failed. No matter.
Try again. Fail again. Fail better.”
-Samuel Beckett

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List of original publications

This thesis is based on the following original publications, which are referred to in the text by their roman numeral.

I Lahdenkari AT*, Suvanto M*, Kajantie E, Koskimies O, Kestilä M, Jalanko H. Clinical features and outcome of childhood minimal change nephrotic syndrome: is genetics involved? Pediatr Nephrol. 2005 Aug;20(8):1073-80.

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II Suvanto M, Jahnukainen T, Kestilä M, Jalanko H. Single Nucleotide Polymorphisms in Pediatric Idiopathic Nephrotic Syndrome. Int J Nephrol. 2016 vol. 2016, Article ID 1417456. doi:10.1155/2016/1417456

III Suvanto M, Patrakka J, Jahnukainen T, Sjöström P-M, Nuutinen M, Arikoski P, Kataja J, Kestilä M, Jalanko H. Novel NPHS2 variant in patients with familial steroid resistant nephrotic syndrome with unusual course of the disease. Clin Exp Nephrol. 2016 Aug 29. PubMed PMID: 27573339

IV Suvanto M, Jahnukainen T, Kestilä M, Jalanko H. Podocyte proteins in congenital and minimal change nephrotic syndrome. Clin Exp Nephrol. 2015 Jun;19(3):481-8.

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

Abbreviations

ACTN4	α-actinin-4
AD	Autosomal dominant
AR	Autosomal recessive
ARHGDIA	RHO GDP-dissociation inhibitor 1
CD2AP	CD2-associated protein
CI	Confidence intervals
CNF	Congenital nephrotic syndrome
DAD	Diaphanous autoregulatory domain
DID	Diaphanous inhibitory domain
EDTA	Ethylenediaminetetraacetic acid
EMP2	Epithelial membrane protein two
ESL	Endothelial surface layer
ESRD	End-stage renal disease
FPE	Foot process effacement
FSGS	Focal and segmental glomerulosclerosis
GBM	Glomerular base membrane
GC	Glucocorticoid medication
GFB	Glomerular filtration barrier
GLCCI1	Glucocorticoid-induced transcript 1 gene
GR	Glucocorticoid receptor
GWAS	Genome wide association study
ILK	Integrin-linked kinase
INF2	Inverted formin 2
IQR	Interquartile range
IS	Immunosuppressive drug
KTx	Renal transplantation
LAMB2	Laminin β2 chain gene
LD	Linkage disequilibrium
LMX1b	LIM homeobox transcription factor
MCNS	Minimal change nephrotic syndrome
mDia	Member of diaphanous formin subfamily of actin-regulating proteins
NPHS1	Nephrin-coding gene
NPHS2	Podocin-coding gene
MDR1	Multidrug resistance gene
MIF	Macrophage migration inhibitory factor
NEPH1	Neph1 coding gene
NGS	Next generation sequencing
NHK	Normal human kidney
NR3C1	Nuclear receptor subfamily 3, group C member 1

NS	Nephrotic syndrome
N-WASp	Neuronal Wiskott-Aldrich syndrome protein
OD	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PLCE1	Phospholipase C epsilon 1SD
SD	Slit diaphragm
SNP	Single nucleotide polymorphism
SRNS	Steroid-resistant nephrotic syndrome
SSNS	Steroid-sensitive nephrotic syndrome
TRPC6	Calcium transporter transient receptor cation channel, subfamily C, member 6
WGS	Whole genome sequencing
WT1	Wilms' Tumor 1
ZO-1	Zona occludens -1

Abstract

The nephrotic syndrome (NS) is characterized by massive proteinuria, edema and hypertriglyceridemia. It can appear as a primary or a secondary disease, idiopathic or familial, monogenic or complex, responsive to medication or progressing inevitably towards end-stage renal disease. As the manifestations of the disease are varied so is the etiologies behind it and still much remains to be discovered. However, structural compromise can be observed in the glomeruli of the NS patients, especially in podocytes, specialized epithelial cells of the glomerular filtration barrier. When causative genetic variants are found they are predominantly in the genes coding proteins involved in the structure and function of the podocytes. To date, over thirty podocyte protein-coding genes have been implicated.

In this study we looked into genetic and cellular mechanisms in the podocyte underlying different forms of NS: congenital nephrotic syndrome of the Finnish type (CNF), steroid sensitive nephrotic syndrome (SSNS) and steroid resistant nephrotic syndrome (SRNS). Specifically, we used CNF kidney samples to study how the different compartments of the podocyte are affected by the lack of nephrin, characteristic structural component of the unique cellular junction of the podocytes (slit diaphragm (SDs)). We also looked into genetic variation in the podocyte protein coding genes in the Finnish SRNS patients to map out the variant spectrum and to see if this population shows difference to other populations. In addition, we aimed to find genetic and clinical markers predicting the course and severity of SSNS, which would have significant clinical value.

The analyses were carried out using an array of molecular biological methods. Protein expression was studied using immunohistochemistry and light microscopy, genetic variants with PCR and sequence analysis. In addition, immunoprecipitation and western blot analyses were carried out to study the functionality of a particular variant. Genome wide sequencing analysis was carried out to gain wider perspective on the variant spectrum in NS patients.

Our results showed that the lack of nephrin leads to widespread effects in the podocyte on protein level, especially on the expression of other proteins of the SD. In other compartments of the cell (basal membrane, actin cytoskeleton, apical membrane) the effect was clear but considering the profound structural damage seen in CNF the orders of magnitude of the observed changes were modest.

We also showed that the analyzed clinical features or variants in these key genes coding podocyte proteins cannot reliably predict severity of SSNS. Children with

difficult disease (multiple relapses, dependence on steroids) are more likely to suffer from NS in adulthood but other correlations between early patient features and prognosis could not be made. Single nucleotide polymorphisms (SNP) analysis of children with idiopathic NS revealed that variants in some genes (e.g. *MDR1*) may be useful in predicting responsiveness to steroids but the correlation is not sufficiently strong to warrant routine clinical testing. We found few causative variants in patients with idiopathic SRNS but did uncover a *de novo NPHS2* variant co-segregating with familial dominant SRNS and unusual course of the disease.

Healthy function of the podocyte relies on interaction and communication between multitudes of protein components in the different compartments of the cell. If any of these components are faulty it may cascade into widespread podocyte damage and proteinuria. The precise nature of the defect may play a significant role in the disease phenotype and not only the function of the damaged gene but also the nature of the variant ought to be considered when carrying out genetic analysis.

Not in all cases causative variants or precise faulty structures can be identified. The genetic factors behind complex traits remain elusive, and even though some pieces of the puzzle have been found, in small part by this study, much is still to be discovered.

1. Review of Literature

1.1 Structure and function of the Nephron

1.1.1 An Overview

Kidneys are two bean-shaped organs of the vertebrates, located at the rear of the abdominal cavity each side of the spine. Their key role is to produce urine, which serves several regulatory and homeostatic functions. These include the removal of excess water, organic molecules, and other waste products of metabolism as well as regulation of electrolytes, maintenance of acid-base balance and regulation of blood pressure via maintaining the salt and water balance.

The functional unit of the kidney is nephron and there are approximately one million nephrons in each human kidney (1). In nephron, under pressure, blood is filtered first into primary urine and then further refined into actual urine that is collected and expelled from the kidney into the bladder (2).

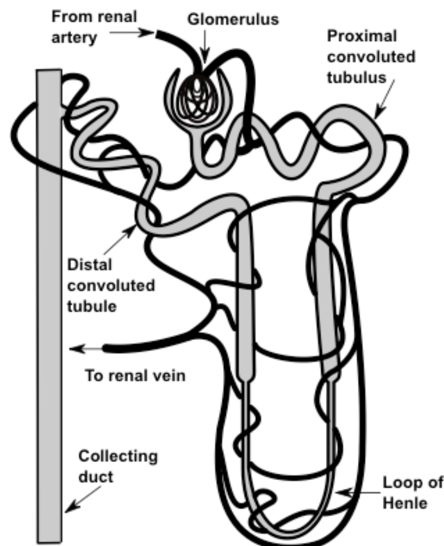

Figure 1 The Nephron

The nephron is composed of two compartments: the glomerulus and the tubule (Figure 1). The function of the glomerulus is to conduct the primary urine filtration, to ensure dependable passing of small solutes while retaining blood cells and proteins the size of albumin and larger in the circulation. The tubule reabsorbs water and small molecules such as electrolytes, peptides and organic molecules from the primary urine based on the needs of the body and thus defines the final concentration and volume of urine, which in healthy adults totals to approximately 1-1,5 liters per day.

In the glomerulus the afferent arteriole branches into a tuft of small capillaries through the walls of which the plasma is filtered (Figure 2). Surrounding the tuft is the expanded end of the proximal tubule, Bowman's capsule. Inside of the capsule is the urinary space, Bowman's space, which gives into the proximal convoluted tubule (2).

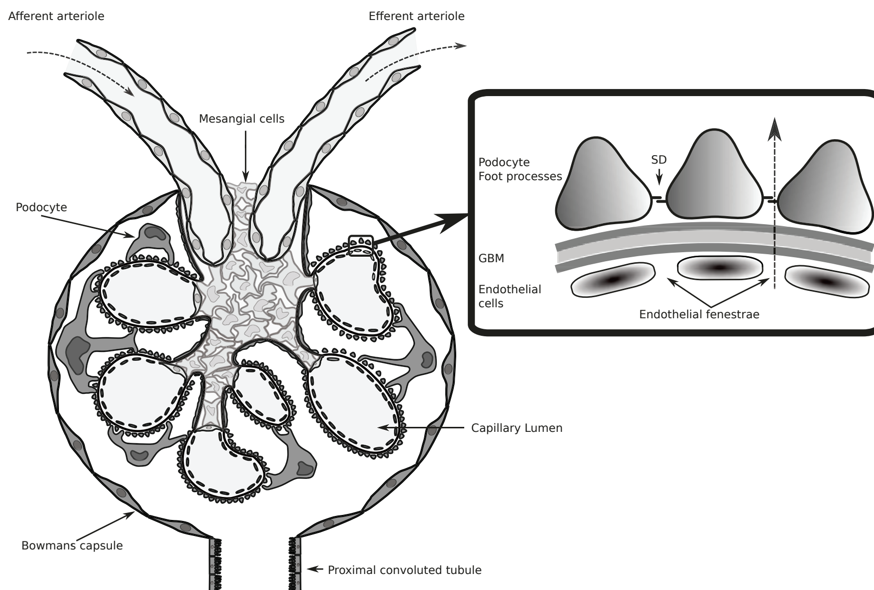

Figure 2 The glomerulus. Adapted from the image of Somlo and Mundel (3)

The tubule is divided into segments that differ structurally and functionally: the walls of different segments have different cell types to accommodate the reabsorption of different substances. Proximal tubule is responsible for the most reabsorption of water as well as the reabsorption of small proteins, amino acids, glucose, and electrolytes. The loop of Henle establishes and maintains osmotic gradient in the renal medulla: its thin descending limb is freely permeable to water and ions while thin ascending limb is much less permeable to water but

very permeable to salts and thick ascending limb is completely impermeable to water and actively transports salts from the filtrate. The distal tubule has two portions: the straight portion also plays a role in establishing osmotic gradient by being impermeable to water while removing salt, and the convoluted portion is responsible for the removal of calcium from the lumen as well as the remaining sodium and chloride and it also actively secretes hydrogen and potassium ions into the lumen. The distal convoluted tubule gives into the collecting duct where more water is reabsorbed and the final volume of urine defined.

The collecting duct leads into the renal calyces from where the urine is moved to the ureter that carries it further to the bladder.

1.1.2 Glomerular filtration barrier

The plasma is filtered through the glomerular capillary wall. The barrier between circulation and urinary space has three layers: 1) the fenestrated endothelium, 2) glomerular basement membrane (GBM) and 3) the foot processes of glomerular visceral epithelial cells, podocytes (Figure 3). The salient function of the glomerular filtration barrier is to prevent the loss of blood cells and proteins into the urine, while efficiently and dependably allowing the passage of solutes to be disposed. The glomerular filtration barrier (GFB) functions under tremendous workload as approximately 180 l of primary urine is formed each day. The filtration of molecules is thought to be shape, size and charge selective (4), although the last is still debated (5). Each layer of the barrier has an essential role in the filtration process and their functions are interwoven as they cross talk and act as a whole. Faults in any of the three layers threaten the integrity of the barrier and homeostasis of the body (6).

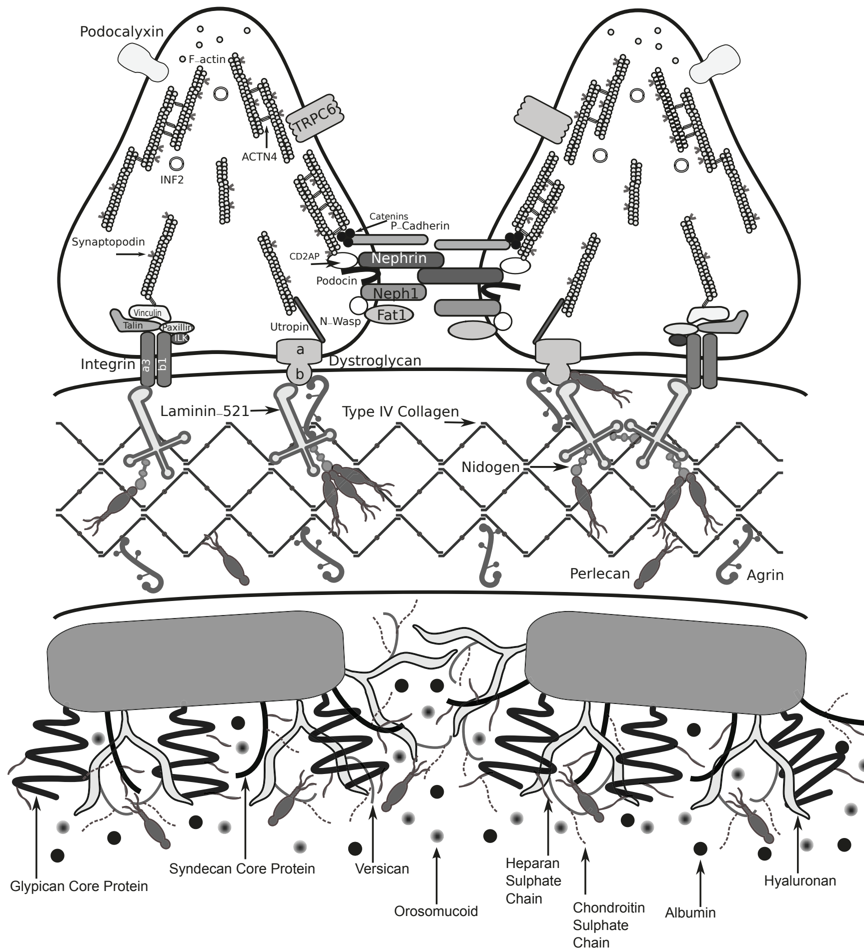

Figure 3 The glomerular filtration barrier. Adapted from the images of Haraldson *et al.* (6), Hallmann *et al.* (7), Machuga *et al.* (8) and Miner (9).

1.1.3 Fenestrated endothelium

The defining feature of the endothelial cells lining the luminal side of the glomerular capillaries is the transcellular fenestration. The fenestrae are 60-80 nm in diameter and occupy 30-40% of the cell surface (10) and do not possess diaphragms (11). They play a salient role in accommodating the high water permeability of the endothelial cells, which is essential for the filtration function of the glomerulus.

Molecules anchored to the endothelial cell luminal surface form polyanionic hydrated mesh called glycocalyx that covers both the fenestral and interfenestral domains. Glycocalyx is a complex structure composed of proteoglycans, glycoproteins and sialic acids (12). It adsorbs plasma molecules including albumin and orosomucoid and secreted proteins such as hyaluronan. Together the glycocalyx and the adsorbed components are known as endothelial surface layer (ESL) (13).

The role of glomerular endothelial cells and ESL in plasma protein retention has been debated. For a long time it was thought to be negligible, mainly due to fenestration pore size being too large to prevent the protein from passing through, but in recent years this attitude has started to change (14, 15). While the diameter of the fenestrae is much larger than that of albumin (60-80 nm vs. 8 μm), biophysical studies show that water and small solutes do not permeate through them as efficiently as expected and the macromolecule permeability of fenestrated capillaries is not higher than that of un-fenestrated capillaries. The glycocalyx is thought to create this resistance to permeation by "plugging" the fenestrae (14, 16, 17).

All layers of GFB are interconnected and function as a whole. Glomerular endothelium and the formation of ESL are regulated by podocyte-secreted proteins including vascular endothelial growth factors and angiopoietin-1 (18-22). Reciprocally, as glycocalyx reacts to shear stress caused by the plasma flow, the behavior of the endothelial cells is modulated to secrete mediators such as nitric oxide that may affect podocyte function (23, 24).

1.1.4 Glomerular basement membrane

The glomerular basement membrane (GBM) is a specialized, sheet-like extracellular matrix located between fenestrated endothelium and podocytes. All basement membranes have the same main components: laminin, type IV collagen, nidogen and heparan sulfate proteoglycan (25). The GBM is unusually thick for a basement membrane and contains particular members of these general protein families (26).

Laminin is a heterotrimeric protein, ubiquitous in basement membranes. It contains three chains (α, β, γ), and all of them have several isoforms that form nonrandom combinations. At least 15 different heterotrimers are known (27). In mature GBM the predominant isoform is laminin-521 (composed of α5, β2 and γ1 chains). Laminin has domains that contain binding sites for cell surface receptors integrin and dystroglycan expressed on the basal cell surface of the podocytes (28).

Type IV collagen is also a trimeric extracellular matrix protein. It is formed of three α-chains. There are six genetically different α-chains that trimerize in specific stoichiometries to form different types of network-forming building blocks. After being secreted into the extracellular matrix, they polymerize and become crosslinked to form a network within the GBM (9). Basement membranes can form without type IV collagen but it is essential for the long-term stability of the structure (29). In mature GBM the major type IV collagen isoform is α3α4α5, although $(\alpha 1)_2\alpha 2$ exists as a minority (30).

There are two nidogen proteins, nidogen-1 and nidogen-2, both common basement membrane proteins. They are at least partially redundant, and knockout mice missing either of two are viable and fertile (31, 32). Nidogen-1 binds both laminin and collagen type IV, and it has been speculated that its role is to connect these two separate networks (33). However, while deletion of both nidogen proteins simultaneously results in perinatal lethality, the basement membranes seem to be able to form in their absence (34). Thus, it is unclear how essential nidogen actually is to GBM.

The major heparan sulfate proteoglycan in GBM is agrin, although it also includes perlecan (35). Agrin has a high negative charge that is thought to majorly contribute to the negative charge of GBM (36). Classically it has been thought that the negative charge of the GBM repels the negative albumin and thus contributes to its retention. However, this view has been challenged, as it has been shown that removal of agrin and perlecan doesn't affect filtration (37, 38). Other study suggested that GBM negativity has more significant role in retention of small, 20-35Å proteins but not in retention of the larger ones (39).

GBM is structured in three layers: between lamina rara interna (close to the endothelial cells) and lamina rara externa (close to the podocytes) lays lamina densa. The laminas rara are rich in heparan sulfate proteoglycans while lamina densa is composed mainly of laminin-521 and α3α4α5(IV) collagen (36).

1.1.5 Podocyte

The visceral epithelial cells, podocytes, lay on the urinary space side of the glomerular capillaries. Podocytes are terminally differentiated, unique cells of particular shape: from the cell body extends primary processes which then divide further into secondary processes and finally into narrow foot processes (Figure 4). The foot processes of adjacent cells are interdigitated and the narrow slit between them is bridged by a specialized sheet-like structure, slit diaphragm (SD). The basal membrane of the foot processes is the only part of the podocyte attached to the GBM, and thus the foot processes along with the SD form the last layers of glomerular filtration barrier. The architecture of the foot processes

is specific and dynamic and maintained by actin cytoskeleton. Each domain of the foot process has its own specialized components and function.

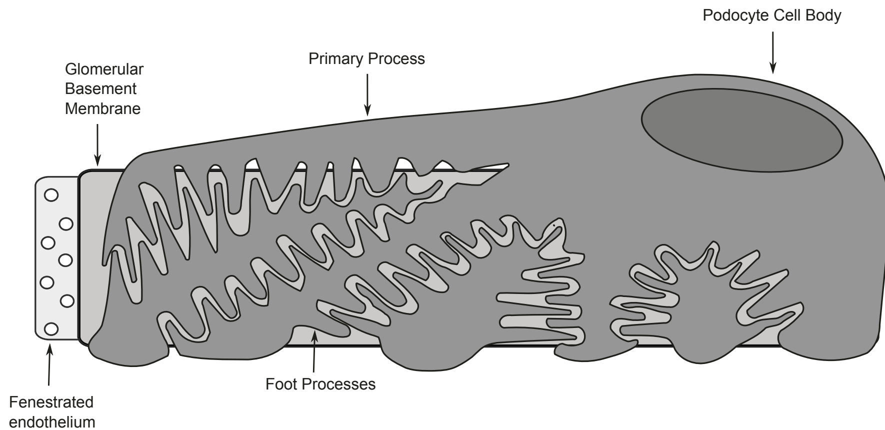

Figure 4 The podocyte on the surface of a glomerular capillary. Adapted from the image of Nilius *et al.* (40).

While the fenestrated endothelium and GBM may block large portion of proteins from passing through GFB, the essential role of podocytes and SD is highlighted by the fact that numerous mutations in genes coding for foot process- and SD-specific proteins lead to massive proteinuria. This speaks for the importance of podocyte signaling properties and communication between the three layers of GFB.

1.1.5.1 Basal and apical membrane domains

Essential driving force in the renal filtration is the hydrostatic and oncotic pressure in the glomerular capillaries. To cope with the pressure the podocyte foot processes must adhere tightly to the GBM. Several basal membrane receptors find their ligands among the components of the GBM and they form the binds that keep the podocytes in their place. The primary adhesion receptors in this task are the integrins, which bind laminin-521 of the GBM. In the cytosol of the foot processes, integrins bind a variety of proteins including signaling and force-transducing molecules that associate with the components of the cytoskeleton (41). As the cytoskeleton is the main stress-baring component of the cell, this tight connection between extracellular matrix and cytoskeleton provides physical reinforcement, which enables the cell to withstand considerable mechanical stress.

The adhesion of podocyte to GBM is regulated by altering integrin activation, clustering and expression in the basal membrane as well as affecting its lateral association with other membrane proteins (42-44). The most abundant integrin in the basal membrane of the podocyte is integrin α3β1. It is thought to play a role in podocyte development as mice lacking the α3 or β1 subunit die soon after birth or already in utero, although the damage is not limited to the kidney. Podocyte-specific deletions of the genes coding for these proteins lead to GBM abnormalities and foot process effacement (45-48). In addition to integrin α3β1, α2β1 and αvβ3 are expressed in the podocyte basal membrane. α2β1 binds collagen IV of the GBM and its deficiency is associated with abnormal GBM-podocyte phenotype in mice (49). The significance of α2β1 is not fully understood but it seems to be minor compared to α3β1. Integrin αvβ3 binds vitronectin and collagen but mice lacking it do not develop overt primary kidney abnormalities so its role in podocyte adhesion to GBM is probably not major (50-52). Curiously, enhanced activation of integrin αvβ3 by soluble urokinase receptor causes foot process effacement and proteinuria in mice and humans (53, 54). Loss of β1-subunit also leads to an increase in αvβ3 activity that may add to pathogenicity of β1 mutations (55).

Another connection between the GBM and the actin cytoskeleton occurs through dystroglycan, a membrane protein that is composed of heterodimeric complex of extracellular α- and transmembrane β-subunits. In GBM, dystroglycan binds proteins such as laminin and agrin, and in cytoplasm, it is interacting with utropin that connects to the actin cytoskeleton (56-58). The relevance of dystroglycan and its function in the podocyte is not yet clear. Its expression is diminished in some forms of experimental and human nephrotic diseases, and it has been suggested to play a role in positioning and organizing the proteins of basal membrane (59). Dystroglycan knockouts die early in embryonic development due to fragility of basement membranes (60), while defective glycosylation of α-subunit is reported to inhibit the binding of laminin and agrin resulting in the flattening of the foot processes (61). However, another study found that podocyte-specific dystroglycan deletion did not result in significant morphological or functional abnormalities (62).

1.1.5.2 Cytoskeleton

The shape and architecture of podocyte foot processes is essential for their integrity and function as a structural component of the filtration. An additional challenge to the podocyte structure is brought by its dynamic nature: podocytes have stress-activated receptors and *in vitro* stretch initiates structural changes (63, 64). Podocyte may also have a contractile role in controlling blood flow, as the foot processes encircle the capillaries (65, 66). All this demands a highly specialized cytoskeleton able to react to surrounding signals and reorganize.

The podocyte cytoskeleton has different composition and function in different parts of the cell. The cell body and major and secondary processes contain vimentin-rich intermediate filaments, while the larger microtubules form organized structures along the major and secondary processes. Intermediate filaments are tension-bearing elements that help to maintain cell shape and rigidity (67). The microtubles are polymers of α and β tubulin that grow and shrink in a highly regulated fashion in response to intra- and extracellular signals. They play an essential role in foot process formation (68, 69) and carry out multiple functions including regulation of cell motility, vesicular transport, the maintenance of cell shape and polarity as well as organization and positioning of membrane organelles (67).

The podocyte foot processes contain long actin fiber bundles that run cortically and continuously from one foot process to the next (70). Adaptor proteins connect the actin cytoskeleton to the intercellular space and the SD as well as to the basal membrane and the GBM (71).

Actin organization and dynamic reorganization is an integral tool in regulating the shape and movement of podocytes, as actin filaments bring them internal mechanical support, tracks for movements of materials, and force to drive cell motility. Monomeric, globular actin is soluble but as it polymerizes it forms insoluble filaments that have considerable mechanical strength (67, 72). Actin can exist as bundles or as networks, and over hundred different proteins tightly regulate all its processes including initiation of new filaments from monomers and adding branch to existing filaments. Many of these proteins are slit diaphragm related, including nephrin, CD2-associated protein (CD2AP) and calcium transporter transient receptor cation channel, subfamily C, member 6 (TRPC6).

α-actinin-4 (ACTN4) is an actin-binding protein with strong expression in podocytes that cross-links actin filaments into contractile bundles (73). Through adaptor proteins such as densin and integrin-linked kinase (ILK), it is also a part of the interaction chains connecting the actin cytoskeleton to the SD and basal membrane. It even interacts directly with integrin α3β1 (70). α-actinin-4 is regulated by extracellular signals that lead to its phosphorylation and decrease its affinity to actin. Mutations in α-actinin-4 cause nephrotic syndrome and are centered on the actin-binding domain. These mutations cause an increase in the α-actinin-4 binding-affinity to actin and change mechanical properties of the cytoskeleton (74).

Inverted formin 2 (INF2) has the ability to both polymerize and depolymerize actin and this function gives it an important role in the regulation of cytoskeletal remodeling (75). Mutations in INF2 cause nephrotic syndrome and the majority of known mutations are located within the diaphanous inhibitory domain that

mediates the INF2 autoinhibition through interaction with the C-terminal diaphanous autoregulatory domain (76, 77).

Proline-rich, actin-associated protein, synaptopodin, has an essential part in regulating foot process shape and motility. Synpatopodin binds ACTN4 and regulates its actin-bundling activity, and adaptor protein CD2AP that binds essential SD component nephrin (78, 79).

1.1.5.3 Slit Diaphragm

Slit diaphragm is a complex, sheet-like structure composed of numerous proteins. It bridges the gap between adjacent foot processes and is the last component of the filtration barrier. Besides an essential structural component of the GFB, the SD is a platform for signal transduction from the extracellular space into the intracellular, and to the actin cytoskeleton and thus plays a pivotal role in dynamic actin remodeling and in altering the foot process architecture, as well as in several other cellular functions (80). Conditions that lead to the breakdown of the SD result in heavy proteinuria.

SD is localized to the lateral membrane of the foot processes, in the lipid rafts, particular microdomains of cell membrane rich in cholesterol, glycosphingolipids and glycosylphosphatidylinositol -anchored proteins (81, 82). The lipid rafts recruit and cluster membrane proteins in dynamic and selective manner and thus provide molecular frameworks for numerous biological processes including endocytosis, exocytosis, cell adhesion, and signal transduction (82). However, the role of lipid rafts in these processes is not to be just a platform for molecules: often the function of the proteins depends on their association with lipid rafts (83).

A characteristic component of the SD is nephrin. It is a 185 kDa-transmembrane protein with eight extracellular immunoglobulin domains, one fibronectin motif and a short cytoplasmic tail (Figure 5). It forms a homodimer with a nephrin molecule from a foot process on the other side of the gap (84). This nephrin bridge is thought to be the backbone of the SD; missing or displaced nephrin leads to dissolved SD (85). In addition to being an important structural component of the SD, nephrin also has a function in signal transduction. In its cytosolic part nephrin has six conserved tyrosine residues that are phosphorylated by Src family kinase Fyn (86). Through adaptor proteins phosphorylated nephrin interacts with actin cytoskeleton and affects several cellular processes including cytoskeletal reorganization, cell growth and survival. Missing nephrin causes nephrotic range proteinuria already in utero (87).

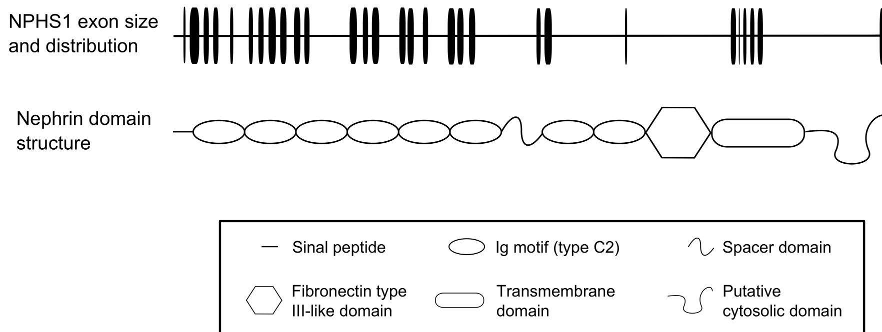

Figure 5 The structure of *NPHS1* gene and nephrin protein

Neph1 is a founding member of family of proteins homologous to nephrin. Their precise function is not yet fully understood but along with Neph1, Neph2 and 3 are expressed in podocyte. With five Ig-domains and no fibronectin motif the Neph proteins are significantly smaller than nephrin, too small probably to reach over the slit gap to form homophilic interaction in trans configuration (88). However, homophilic interaction in cis conformation is suggested, as is heterophilic interaction with nephrin in both cis and trans configurations (89, 90). Nephrin/Neph1 interaction is an important determinant for glomerular permeability (91), and it is also involved in the signal transduction to induce actin polymerization (92). Based on experiments in Drosophila melanogaster, it is speculated that Neph1 is involved in patterning of the SD and in signaling. Neph1 knockout mice die perinatally (93). However, unlike nephrin, expression of which is limited to kidney, Neph1 is expressed in multiple organs, including kidney, brain, placenta, gut, heart and lung (94). So far podocyte-specific Neph1 knockout studies have not been published.

Protocadherin Fat1 is also located in the SD. *In vitro* studies indicate that mammalian Fat1 is involved in planar cell polarity and directed actin-dependent cell motility (95). Although its precise function in the SD is not yet fully understood, these functions could make it salient regulator of foot process structure and dynamics. Loss of Fat1 leads to massive proteinuria and early perinatal lethality in mice (96)

Another SD-located protein P-cadherin is a member of cadherin superfamily that is known for mediating homophilic cell-cell interactions. This could be its role in SD as well. However, little evidence exists on P-cadherin's function in the SD. P-cadherin deficient mice do not seem to have an aberrant renal phenotype (97) and a study was unable to show *in vivo* or *in vitro* association of P-cadherin to either nephrin or Neph1 (98). More is known of P-cadherin’s interaction with

adaptor protein β-catenin that stabilizes the SD and maintains its integrity (99, 100).

In addition to the proteins with extracellular domains that physically form the protein bridge, essential for the integrity and function of the SD are the adaptor proteins that connect the structure to the intracellular domains of the foot process. Podocin is located in the cell membrane at the insertion site of the SD. It has one transmembrane region and two cytosolic ends, which leads to a hairpin like structure (101) (Figure 6). The cytosolic ends bind proteins, including nephrin and Neph1, and it is presumed that the key function of podocin is to anchor nephrin to correct location in the lipid rafts (102). In addition to SD proteins podocin binds and regulates cation channel TRPC6, which is involved in the mediation of pressure sensation in the podocytes (103). Podocin is also tightly associated with CD2AP.

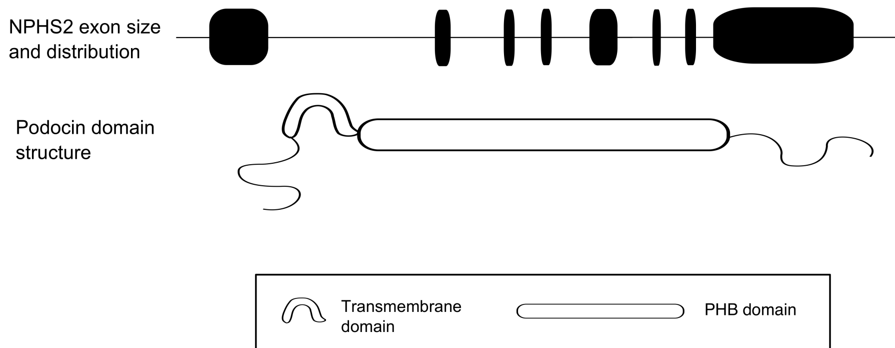

Figure 6 The structure of *NPHS2* gene and podocin protein

CD2AP is expressed primarily in the cytosolic side of SD. It binds both nephrin and podocin and also the actin cytoskeleton forming a direct connection between the SD and the cytoskeleton. It is also involved in the SD signal transduction function. CD2AP and nephrin interact with a subunit of phosphoinositide 3-kinase (PI3K), which in turn activates intracellular Akt kinase pathway. This is needed in regulation of actin dynamics and cell survival (104). In animal models as well as in few human patients mutations in CD2AP gene have been associated with proteinuria.

During development, the junction between podocyte foot processes evolves from a tight junction to a modified adherens junction (SD) and contains protein components from both types (98, 105, 106). Zona occludens -1 (ZO-1) is a membrane protein that is characteristic for cytoplasmic surface of tight junctions in epithelial and endothelial cells. In podocytes, it is found in the in

insertion of the SD and is linked to the actin cytoskeleton forming another connection between the two domains. In an animal model, redistribution of ZO-1 was associated with ultrastructural changes in the foot processes and apical displacement of SD (107).

There are two Nck proteins in the podocyte, Nck1 and Nck2, with highly redundant functions. Mouse studies show that Nck proteins are required for normal filtration barrier development as well as for its maintenance in mature state (108, 109). The Nck proteins have a SH2 domain, that is capable of interacting with phosphorylated tyrosine residues, and SH3 domains, which can recruit other proteins. In SD, the SH2 domain of Nck interacts with the several Src-family kinase Fyn-phosphorylated tyrosine residues in the cytosolic end of nephrin. The SH3 domains of Nck bind neuronal Wiskott-Aldrich syndrome protein (N-WASp) and N-WASp, in its turn, activates Arp2/3 complex and cortactin, thus connecting nephrin-Nck complex to the actin cytoskeleton (108). Nck and its actin-regulating associates are recruited to phosphorylated nephrin when rapid actin polymerization and cytoskeletal reorganization is required during development and injury repair (110). In a steady state, nephrin-Nck interaction may be rarer compared to predominant nephrin-CD2AP interaction (111, 112).

β-catenin is adaptor protein that forms a complex with P-cadherin to stabilize the adherens junction and maintain its integrity as well as link it to the cytoskeleton (99, 100). In association with Wnt β-catenin is also a key regulator of gene expression via binding to transcription factors. Binding Wnt ligand leads to dephosphorylation and stabilization of β-catenin, which allows it to translocate into the nuclei and stimulate the transcription of Wnt-targeted genes (113). In podocytes, Wnt/β-catenin signaling pathway plays a salient role in cell adhesion, differentiation and survival/apoptosis (114).

1.2 Proteinuria

Leakage of protein to urine is often the first sign of many renal conditions, and functions as an independent risk factor for development of end stage renal disease. It is also associated with non-renal conditions, such as cardiovascular disease, and is a predictor of all-cause mortality rates. While some reports have looked into the role of the proximal tubule and its protein uptake in the development of proteinuria, the vast majority of studies have placed the blame on the GFB. Indeed, the genetic injuries underlying many proteinuric kidney diseases are found in proteins involved in the GFB function, especially in those located in the podocyte foot processes.

1.2.1 Endothelium and proteinuria

While several inherited kidney diseases have found their genetic cause in the components of GBM, podocyte SD and actin cytoskeleton, damage to endothelial cells may play a role in acquired kidney disease.

Mechanisms that lead to loss of function in glomerular endothelium include reduction of density of fenestrae and alteration in their size, swelling of the endothelial cell and encroachment of the capillary lumen and thus increase in the shear forces (112). However, the key mechanism to induce proteinuria seems to be alterations in the glycocalyx of the endothelial cells. One study showed that an injection of lipopolysaccharide (LPS) (used as a model for sepsis) caused injury to endothelium resulting in acute kidney failure in mice. Even though the LPS-induced damage included albuminuria, no alteration in podocytes (including morphology, SDs, number, size etc.) was seen in electron microscopy (116). LPS injection resulted in an increase in heparanase that degrades heparan sulphate glycoproteins of the glycocalyx and in a reduction of sialic acid containing proteoglycans in the ESL (116). Other studies found that reduction of ESL hyaluronan by hyaluronidase and inhibition heparan sulphate glycosaminoglycan synthesis by high glucose lead to increased albumin permeability (117, 118).

Other conditions that damage glomerular endothelium and lead to proteinuria include thrombotic thrombocytopenic purpura, diarrhea-associated hemolytic uremic syndrome, atypical HUS and preeclampsia (115, 119-122).

1.2.2 GBM and proteinuria

What and how large a role GBM plays in protein retention in healthy kidney is still unclear but genetic defects in its key components are known to result in proteinuric conditions. Mutations in *LAMB2*, gene coding for laminin β2 chain, leads to Pierson syndrome, a congenital nephrotic syndrome (CNS) associated with variable ocular and neurological manifestations (123). Pierson Syndrome follows autosomal recessive (AR) inheritance pattern and a majority of the causative mutations are truncating ones that are distributed equally to all exons (124). The truncating mutations lead to the reduction of β2 chain and thus to reduction of laminin-521 trimer, faulty GBM and proteinuria (125). Missense mutations and small inframe deletions in LAMB2 are reported in patients with milder disease phenotype (126, 127). The missense mutations are clustered in N-terminal globular laminin domain, important in intermolecular interactions (124).

Mutations in the genes coding for any of the collagen IV α3, α4 or α5 chains can result in damaged GBM. Depending on the type of mutation, the damage can

vary from mild thin membrane disease to severe Alport syndrome. The thin membrane disease follows autosomal dominant (AD) inheritance pattern and is caused in 40-50% of patients by heterozygous null mutations in *COL4A2* or *COL4A4* (coding for α3 and α4 chains of type IV collagen, respectively) (9). Patients express hematuria, but the disorder does not usually progress or need treatment, although elevated risk of proteinuria and hypertension exists (128). In a homozygous state, the same mutations cause AR Alport syndrome characterized by hematuria, eventual renal failure and lamellated GBM as well as hearing loss and ocular abnormalities (128) Majority of patients also suffer from proteinuria. AR mutations of *COL4A3* and *COL4A4* are found in 15% of Alport syndrome patients, 85% have mutations in X-chromosomal *COL4A5* (128).

It has been thought that the contribution of GBM to GFB is charge selectivity based on the negative charge of both GBM and albumin. However, in recent years this view has been questioned. For instance, one study showed that the deletion of negatively charged heparan sulphate proteoglycan agrin dramatically reduced the anionic charge of GBM but did not affect albumin filtration (129). Another study suggested that charge plays a minor role and affects mainly small pores and thus small proteins of 20-35Å (39).

1.2.3 Podocytes and proteinuria

Podocytes are essential for the filtration barrier integrity and their injury typically results in proteinuria. Mutations in many podocyte proteins, especially in those involved with the SD and actin cytoskeleton regulation are mutated in different forms of nephrotic syndrome (NS).

In response to injury it is characteristic for podocytes to undergo vast changes in appearance. The most general type of change is called foot process effacement (FPE), which is seen in most cases of proteinuric kidney diseases from mild steroid sensitive nephrotic syndrome (SSNS) to severe CNS and experimental animal models. In its early stage it is characterized by loss of the interdigitated structure of the foot processes that retract into short cell projections with undefined shape (130). The number and width of the slit pores appears to be decreased and SDs are disrupted: lost or displaced from their usual position or replaced with other type of junction, typically occludens-type junction (131-133). Although the changes in the podocyte structure in FPE are drastic it is notable that the state is reversible. In experimental models FPE and its reversal have been seen in mere minutes after induction (134).

As the FPE progresses, the foot processes merge first with the primary processes resulting in flattened, disc-like appearance of the projections covering the GBM, and then with the cell bodies. At this stage the cell bodies no longer float in the

filtrate of the Bowman's space but are broadly attached directly to the GBM and in many places the subpodocyte space is mostly disappeared (135). In the final stage of FPE development, the cytoskeleton is rearranged into a basal cytoskeletal "mat" in close proximity to the GBM. It is composed of highly organized densely interwoven microfilaments crosslinked by α-actinin positive densities (135, 136). α-actinin appears to play an integral role in this cytoskeletal rearrangement, as in rats with puromysine aminonuclease nephrosis its expression is reported to be induced before FPE and proteinuria (137). The intact podocyte foot processes are anchored to the GBM by integrins and especially α3β1-integrin. In a FPE model in cultured podocytes, α3β1-integrin is downregulated while αvβ3-integrin is upregulated. It appears that αvβ3-integrin increases the cultured podocytes ability to withstand mechanical stress (138).

One suggestion is that instead of a primary injury FPE is actually protective response to injury, an attempt to prevent podocyte detachment (133). There are about 800 podocytes per glomerulus and even healthy glomeruli lose podocytes during a lifetime. In normal circumstances the rate of excretion is low enough that a person is sufficiently sustained until advanced age. Several studies suggest that in NS as well as in animal models the rate of depletion increases dramatically and is preceded by FPE. Podocytes are terminally differentiated postmitotic cells that cannot proliferate or be generated from other glomerular cells (133, 139). As podocytes are essential for kidney function it is possible that mechanisms including FPE have developed to prevent their loss.

Traditionally, the major mechanism for podocyte loss in progressive glomerular disease is thought to be apoptosis (140-142). This view has been challenged and it is suggested that podocytes in situ detach as viable cells that are lost due to podocytes inability to stay attached to GBM (133, 143). However, while many do think podocytopenia is an important determinant of a late stage glomerular injury, other studies have found no evidence of the phenomenon (144, 145).

1.3 Proteinuric kidney diseases in children

Nephrotic syndrome (NS) is characterized by massive proteinuria (>40mg/h/m2), hypoalbuminemia (<25g/l), hyperlipidemia and edema (146). While NS often occurs as a secondary feature to systemic a condition to glomerulonephritis or vasculitis, here we concentrate on primary childhood-onset NS diseases. Primary NS can occur as an inherited, familial condition or as an idiopathic disease. Different NS entities are typically categorized based on responsiveness to steroid medication and histology. Typically, sensitivity to steroids is associated with minimal histological changes and thus it is often synonymously called minimal change nephrotic syndrome (MCNS). However, in some cases SSNS is associated with more severe histology, such as focal and

segmental glomerulosclerosis (FSGS) or diffuse mesangial sclerosis (DMS) that are usually associated with steroid-resistance. Likewise, sometimes steroid-resistant NS (SRNS) has minimal histological changes. It is possible that in some cases the severity of the histology depicts more the stage of development of a disease than separate disease entities. However, it is reported that only 1-3% of patients who initially respond to corticosteroid treatment develop steroid resistance later (147).

As the clinical manifestations of different forms of NS are varied so are the genetic factors underlying them. Variants in over 30 different genes have been associated with different primary and secondary forms of NS (Table 1). However, only a portion of NS cases have determined causative factor; the majority remain unclear.

Table 1 The proteins and genes variants in which lead to NS

	Protein	Gene	Condition	Inheritance	Histology	Reference
SD related	Nephrin	*NPHS1*	CNF, SRNS	AR	Microcystic tubule dilation, progressive MS (CNS), MPGN, MCD, FSGS (SRNS)	(87)
	Podocin	*NPHS2*	SRNS, CNS	AR	FSGS, MCNS	(148)
	PLCe1	*PLCe1*	DMS, SRNS	AR	FSGS	(149)
	CD2AP	*CD2AP*	SRNS	AD/AR	FSGS	(150)
	TRPC6	*TRPC6*	SRNS	AD	FSGS	(151)
Actin reg.	a-actinin-4	*ACTN4*	SRNS	AD	FSGS	(152)
	Inverted formin-2	*INF2*	SRNS	AD	FSGS	(76)
	NMMHC-A	*MYH9*	FSGS	-	FSGS	(153)
	Arhgap24	*ARHGAP24*	SRNS	AD	FSGS	(154)
	Non-muscle class I myosin 1e	*Myo1E*	SRNS	AR	FSGS	(155)
	RhoGDP dissociation inhibitor α	ARHGDIA	CNS	AR	DMS	(156)
	Anillin	ANLN	SRNS	AD	FSGS	(157)
Basal membrane	Integrin α3	ITGA3	CNS, interstitial lung disease, epidermolysis bullosa	AR	FSGS	(158)
	Integrin β4	ITGB4	CNS, epidermolysis bullosa, pyloric atresia	AR	FSGS	(159)
	Epithelial membrane protein 2	EMP2	SSNS, SRNS	AR	MCNS	(160)
GBM	Laminin β2	*LAMB2*	Pierson's syndrome	AR	DMS, FSGS	(123)
Transcription factors	WT1	*WT1*	Denys-Drash, Frasier, SRNS	AD, AR	DMS, FSGS	(73, 161)
	LIM homeobox transcription factor 1b	*LMX1B*	Nail-Patella syndrome	AD	FSGS	(162, 163)
	SMARCA-like protein	*SMARCL1*	Schimmke immuno-osseus dysplasia	AR	FSGS	(164)
Mitochondria	tRNA_LEU	*MTTL1*	MELAS	?	FSGS	(165)
	COQ6	*COQ6*	SRNS, sensorineural deafness	AR	FSGS	(166)
	Parahydroxybenzoate-polyprenyltransferase	*COQ2*	CoQ10 deficiency	AR	Collapsing glomerulopathy	(167)
	Prenyl diphosphate synthase subunit 2	*PDSS2*	CoQ10 deficiency/Leigh syndrome	AR	FSGS	(168)
Others	Lysosomal integral membrane protein type 2	*SCARB2*	Action myoclonus-renal failure	AR	-	(169)
	Zinc metallo-proteinase STE24	*ZMPSTE24*	Mandibuloacral dysplasia	AR	-	(170)
	?	*GMS1*	Galloway-Mowat syndrome	AR	DMS, FSGS	(171)
	Phosphomannomutase 2	*PMM2*	Congenital defects of glycosylation	AR	Collapsing glomerulopathy	(172)
	b-1,4-mannosyltransferase	*ALG1*	Congenital defects of glycosylation	AR	FSGS	(173)
	GLEPP1	*PTPRO*	SRNS	AR	FSGS	(174)
	Glypican-5	*GPC5*	SRNS	AR	FSGS	(175)

1.3.1 CNF

Congenital nephrotic syndrome of the Finnish (CNF, NPHS1) type is a rare hereditary pediatric disease that is more common in Finland than elsewhere, with a frequency of approximately 1 in 8200 live births. Around the world, small populations exist where the disease has even higher frequency. For example among a subgroup of old order Mennonites in Lancaster County, Pennsylvania the frequency rises to 1 in 500 (176).

CNF is characterized by massive proteinuria already in utero and severe nephrotic syndrome after birth. The disorder develops to end-stage renal disease (ESRD) very early in life and is lethal without intervention. No medicinal cure exists, although symptoms may be alleviated with albumin infusions that are commenced daily. In Finland, bilateral nephrectomy is carried out when the child weighs 7 kg, followed by dialysis. Renal transplantation is performed when the patient weighs approximately 10 kg and is 1-2 years old (177, 178).

CNF follows AR inheritance pattern and is caused by mutations in the key SD component nephrin, encoded by *NPHS1*, which is a large gene of 26kb and 29 exons located at chromosome 19q13.1. More than 200 different mutations have been reported worldwide, while in Finland 97% of all cases are caused by two particular mutations, Fin-major and Fin-minor (87). Sixty percent of the Finnish patients are homozygous for Fin-major, a deletion of two nucleotides in exon 2 (c.121_122delCT) that leads to a premature stop-codon and truncated protein product of only 90 amino acids instead of the 1241 amino acids of the healthy protein. Fin-minor is a nonsense substitution in exon 26 that leads to a stop-codon and truncated protein of 1109 amino acids (c.3325C>T). The other disease-causing mutations in CNF show great diversity as they include insertions, deletions, nonsense, missense and splicing mutations. While *NPHS1* mutations are predominantly associated with CNF, recent studies have identified certain specific, less severe mutations in patients with FSGS and disease onset at childhood or even adulthood (179).

Reports show that many of the nephrin mutations, including Fin-major and Fin-minor, lead to total absence of nephrin from the SD (85). This is due to the damaged protein getting caught by the cellular quality control mechanisms at the endoplasmic reticulum. This mechanism plays an interesting, integral role in the development of the severe and quite homogenous disease phenotype of CNF caused by variety of different type of mutations, some leading only to single amino acid change in the protein.

1.3.2 SRNS

Steroid resistant nephrotic syndrome (SRNS) accounts for approximately 15% of childhood primary NS globally (180). Typically SRNS is associated with FSGS in light microscopy (181). Electron microscopy (EM) shows foot process effacement as well as loss of foot processes even in the nonsclerotic areas. Loss of podocytes is reported in some adult SRNS patients. Clinical features of SRNS include proteinuria, hypertension and progression to ESRD. It is notable, however, that due to the great etiological heterogeneity behind SRNS considerable variability also exists in its clinical manifestations. As SRNS often progresses to ESRD bilateral nephrectomy and renal transplantation (KTx) is required. In a subset of approximately 33% of the patients the disease recurs after transplantation (182).

There are idiopathic and familial forms of SRNS. While the majority of causative factors behind idiopathic SRNS remain unknown, approximately one third of cases have an underlying genetic injury that plays a role in the pathogenesis of the condition. The lower the age of onset the more likely it is that a causative variant can be found (183-185). Typically, cases where a genetic injury can be found run a lower risk of recurrence after KTx. Familial SRNS may follow either AR or AD inheritance pattern. AR form of the disease often has lower age of onset and more severe course of disease than AD form of the disease, which may manifest only in adulthood. A majority of the genes mutated in SRNS code proteins expressed in podocyte foot processes.

Mutations in *NPHS2*, coding for podocin, lead to AR SRNS and are found in 12-17% of sporadic cases (180). *NPHS2* mutations are the most common genetic cause of SRNS identified in European patients. The 25kb *NPHS2* is located on 1q25-31 and consists of eight exons. Over a hundred disease-causing mutations have been found across the whole length of the gene. SRNS caused by homozygous or compound heterozygous mutations has a quite aggressive course with onset before six years of age and progression to ESRD during first decade of life. Different types of mutations may lead to variance in disease phenotype. Complete loss of podocin function may alter glomerular development and lead to CNS (186, 187). Mutations in C-terminus of the podocin, such as the common European mutation p.R138Q, lead to its retention in the endoplasmic reticulum of the podocytes and inability to take its place in the plasma membrane (188). As podocin plays a role in anchoring other proteins, including nephrin, CD2P and TRPC6, to the correct position in the membrane, retention of podocin may lead to their mislocalization as well. Other mutations are reported to induce podocyte apoptosis instead (188).

Variant p.R229Q has unclear effect on podocin function but it does affect the disease phenotype as patients with heterozygotic p.R229Q together with another pathogenic variant in heterozygotic state lead to significantly higher age

of onset. As healthy individuals have been reported to carry homozygous p.R229Q, it either is not a pathogenic variant itself, or has an incomplete penetrance. One study suggested that the variant is not disease causing, but a modifier of variants in other genes (189). Another study reported that p.R229Q is only pathogenic when it is in a compound heterozygotic state with a pathogenic variant located in the exons 7 or 8 (190).

Mutations in several genes coding for protein associated with actin-cytoskeleton have been discovered in patients with SRNS. While the precise functional effects of most of the mutations are not yet known they may affect the ability of the cytoskeleton to dynamically respond to changes in pressure of the capillary walls (191). CD2AP forms a link between SD and the actin cytoskeleton and mutations in it have been reported in few human patients. Heterozygous mutations cause sporadic SRNS with age of onset between 2-23 years while the one patient with homozygous CD2AP mutation reported so far suffers from more severe infantile form of NS (192-194). Similar pattern is seen in mouse models where CD2P-/- knockouts die at six weeks of age, while CD2AP+/- manifest proteinuria at nine months of age and generally exhibit similar pathology to human SRNS (150, 193)

Missense mutations in ACTN4 gene coding for α-actinin-4 are associated with incompletely penetrant and late-onset AD SRNS (152). ACTN4 mutations in SRNS are only found in 4% of patients with familial SRNS (195). The identified mutations are clustered in the actin-binding domain resulting in increased binding to filamentous actin and formation of protein aggregates in vitro (196, 197). In addition to affecting the cytoskeleton, a mouse model with ACTN4 mutation K255E developed FSGS and activated the endoplasmic reticulum stress response, which results in expression of pro-apoptotic proteins and leads to possible cell death (198, 199).

Mutations in INF2 cause 17% of AD SRNS but are rarely associated with sporadic cases (76, 77, 200). INF2 mutations are also found in patients with Charcot-Marie-Tooth disease and glomerulopathy (201). INF2 interacts with members of diaphanous formin subfamily of actin-regulating proteins (mDias). MDias have formin homology domains that are sites for actin nucleation and polymerization. They also have two regulatory domains: diaphanous inhibitory domain (DID) and diaphanous autoregulatory domain (DAD). In the absence of member A of Ras homolog gene family (RHOA), DID and DAD interact to inhibit actin polymerization. INF2 also has DID and DAD domains and INF2-DID can also bind mDia-DAD and thus modulate mDias potential for actin polymerization (202, 203). Majority of described INF2 variants are heterozygous missense mutations clustered in the exons 2-4, which code for the N-terminus of the DID domain. The mutations may damage the inhibitory function of DID domain and result in overactivation of mDias and unbalanced actin polymerization (203).

Homozygous mutations in *ARHGDIA*, coding for RHO GDP-dissociation inhibitor 1, a regulator of GTP/GDP binding RHO GTPases, have recently been found in an infant with CNS and two siblings with early onset SRNS (204). In podocyte culture, wild type ARHGDIA binds RHOA, RAC and CDC42 and inhibits cell migration (204). Mutated ARHGDIA increases RAC1 and CDC42 activity in vitro (204).

Mutations in *TRPC6* cause AD FSGS. Studies suggest that disease-causing mutations result in overactivation of the channel (151, 205, 206). This theory is supported by a study finding overexpression of TRPC6 in mice with FSGS (207). One possible mechanism for this route to podocyte injury is that TRPC6-mediated calcium influx plays a role in mechanosensation. Increased calcium in the cell activates RHOA and thus affects the actin polymerization of the cytoskeleton as well as downregulate nephrin expression (208).

Mutations in phospholipase C epsilon 1 (*PLCE1*) result in early onset SRNS and the quality of the mutation is reported to affect the disease phenotype as patients with truncating mutations showed histological lesion of diffuse mesangial sclerosis (DMS) while those with missense mutations had FSGS (149). In a mouse model, while PLCE1 knockout had no renal phenotype, enhanced PLC-family signaling lead to podocyte injury and proteinuria (149, 209). PLC- family catalyzes the generation of second messengers to trigger the release of ER's calcium storage pool and activate TRPC6 and thus increase the calcium influx in the cell (209, 210). PLCE1 may also play a role in podocyte differentiation and regulation of actin cytoskeleton and is associated with the SD components such as nephrin and podocin (149, 211, 212).

In addition to proteins involved with SD and actin cytoskeleton, and their regulation, other podocyte-expressed proteins may be injured by genetic mutation in SRNS patients. Mutations in glomerular epithelial protein, GLEPP-1, located at the apical membrane, cause AR SRNS (174). Wilms' Tumor 1 (WT1) is a transcription factor that is essential for the renal development and differentiation. Mutations in *WT1* cause Denys-Drash and Frasier syndromes as well as isolated NS (161, 213). Mutations in LIM homeobox transcription factor *LMX1b* are behind rare AD disorder Nail-Patella syndrome (214). Both of these transcription factors are also involved in the regulation of SD proteins. Mutations in lysosomal protein SCARB2 are found in AR myoclonus-renal failure syndrome (169) while mitochondrial gene *MT-TL1* (coding leucine tRNA) mutation causes SRNS and respiratory chain defect (215). Apolipoprotein L1 mutations have been identified in African American patients with FSGS (216).

1.3.3 SSNS

Steroid sensitive nephrotic syndrome, SSNS, is the most common form of NS in children. It usually first manifests at 2-6 years of age and is twice as likely in boys than in girls. It is characterized clinically by a rapid onset of edema. Most often urinanalysis reveals only proteinuria but some patients may have hyaline or waxy casts and hematuria. While SSNS is usually associated with minimal changes in the glomerular structure in light microscopy, electron microscopy reveals foot process effacement, fewer SDs and reduction in slit pore size (217).

SSNS is less severe than other forms of childhood NS as 85% of patients recover in four weeks of corticosteroid medication. However, SSNS has a tendency to run a relapsing course: about 80% patients have more than one episode and, of those who relapse, approximately half have frequent relapses (more than two relapses in six months or more than four in any one year) or become steroid-dependent (two consecutive relapses during the corticosteroid treatment or within 14 days after its cessation) (218, 219). SSNS may be complicated by infections due to immunosuppressive therapy and thrombosis caused by combination of relative hypovolemia and hypercoagulable state (220).

The etiology of SSNS is still not well understood although several avenues have been researched. Popular proposition is that SSNS has an immunological component as it is associated with abnormal T-cell response and often appears together with disorders with immunological base, such as atopy, autoimmune diseases and lymphomas (146, 221-223). It also often first appears after an infection although the mechanism of this is not known. Recently, especially respiratory tract virus infections are implicated (224, 225). T2-helper cell activation and production of cytokines such as IL4 and -13 (IL4 and IL13) that occurs in allergy, also occurs in animal models of SSNS (226, 227). However, a direct link between immune system and SSNS is yet to be found. On glomerular level, loss of GBM charge has been reported in some patients (228, 229).

An interesting recent entry for causative component in SSNS is angiopoietin-like-4 (ANGPTL4). ANGPTL4 is expressed in several tissues, among these the podocytes, although in a low level in normal state. In a rat model podocyte-specific overexpression of ANGPTL4 leads to features similar to human SSNS: albuminuria, foot process effacement, responsiveness to steroids, as well as reduced charge in the GBM (230). As podocyte-secreted ANGPTL4 binds to the GBM and possibly reduces its charge, its binding sites in the GBM are clustered to areas that show foot process effacement, and glucocorticoid (GC) treatment reduced its expression significantly, Clement *et al.* suggested the protein plays a significant role in the development of SSNS (230). It is yet to be discovered if the same findings can be made in human disease.

As a significant proportion of SRNS patients carry mutations in genes coding for podocyte proteins, these genes are of interest also in SSNS patients. As SSNS is a complex trait it is not likely that a single gene variants would have such an effect as in SRNS, but genetic variants may still have a role in the pathogenesis of disease, causative or modifying. So far, genetic findings in SSNS are scarce. A report by Hinkes *et al.* (149) found two patients with AR NS and mutations in *PLCE1* gene (coding for 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1), one of which responded to cyclosporin and the other to corticosteroids. Interesting new candidate is epithelial membrane protein two (EMP2). Gee *et al.* (160) found recessive variants in its coding gene in three SSNS patients from two families. EMP2 is exclusively expressed in glomeruli and in knockdown mice without any EMP2 expression podocytes exhibited increase in CAVEOLIN-1, which is partially located in the SD. The particular connection between EMP2 variants, CAVEOLIN-1 and SSNS requires more investigation.

1.3.3.1 Genetics of steroid sensitivity

An essential question in NS treatment is to find reliable markers that predict the patient's response to steroid medication. Existence of pathogenic variants in genes coding for podocyte proteins is thought to be a good indicator of steroid resistance although a few exceptions are found as previously mentioned. However, use of these variants as predictors can be onerous as the number of potential genes and variants continues to increase, even though developing techniques ease the burden. More eminent problem is that pathogenic variants are not found in all patients. Other predictors are required and the genetics and pharmacokinetics of steroid responsiveness are still insufficiently understood.

A key component in the effectiveness of GCs is the glucocorticoid receptor (GR). In its inactive state GR is involved in multiprotein complex which, upon binding the GC breaks down. The active GR is transported in the nucleus where it undergoes dimerization, binds specific DNA sequences, the glucocorticoid response elements, and as a monomer, interacts directly with other transcription factors such as nuclear factor kappa B (231-233). Binding of GR to glucocorticoid response elements leads to increased activation of genes coding for anti-inflammatory proteins, such as interleukin-10 and inhibitor of nuclear factor kappa B (232). GR is coded by nuclear receptor subfamily 3, group C member 1 (*NR3C1*) and the involvement of several of its polymorphisms with idiopathic NS (INS) and steroid responsiveness has been studied (233-235). Curiously, while some polymorphisms were associated with reduced GC sensitivity, others lead to its increase (233, 234, 236, 237)

Pro-inflammatory cytokines may also be involved in the formation of the GC response. One such cytokine is macrophage migration inhibitory factor (MIF). MIF is expressed mainly by T-cells but also by epithelial and endothelial cells in several tissues, including the podocytes (238). It is involved in securing innate

immune resistance of the organism but, as the range of action of the cytokines is broad, it is also a mediator in many inflammatory and autoimmune diseases, such as septic shock, ulceratis colitis, pneumonia, tumors and glomerulonephritis (239-241). While cytokines in general are suppressed by GCs, MIF interestingly seems to be induced by GCs and leads to inhibition of their activity (241, 242). Thus, therapies aiming at inhibition of MIF may be a potential way of enhancing corticosteroid responsiveness. Several polymorphic variants in MIF gene have been analyzed in many related diseases, including in NS. The minor allele in SNP –173G/C (rs755622) in MIF gene promoter was shown to be more frequent in steroid resistant patients (239, 240, 242).

Rather new, potential target in association with steroid responsiveness is glucocorticoid-induced transcript 1 gene (GLCCI1), which is involved in steroid resistance in asthmatic patients (243). GLCCI1 is expressed in podocytes and knockout mice develop podocyte injury and proteinuria (244). Analysis of the asthma-associated SNPs has been carried out in Korean NS patients but no involvement was observed (245). The cellular role of GLCCI1 is yet to be clarified.

P-glycoprotein (P-gp), coded by multidrug resistance gene *MDR1*, is a transporter of xenobiotics involved in elimination and intracellular concentration of many drugs including corticosteroids. Wasilewska *et al.* (246) measured P-gp expression in CD3 leukocytes in peripheral blood and found that SSNS patients had increased expression compared to healthy controls during relapses (before, during and 2 months after corticosteroid treatment), and the expression level was comparable to the corticosteroid dosage. P-gp expression level in the lymphocytes was also found to be significantly elevated in frequently relapsing and steroid-dependent patients compared to infrequently relapsing (247). Several studies have looked into the association of MDR1 polymorphic variants and NS as well as steroid responsiveness and found three variants (G3435T, G2677T/A, C1236T) to be related to both NS and to steroid responsiveness (248-251). These variants are thought to be associated with enhancement in MDR1 expression.

Other potential factors engaged in the development of steroid resistance include subfamily 3A of human cytochrome P450, which plays a significant role in drug metabolism (233, 251), as well as pro-inflammatory cytokine tumor necrosis factor-alpha and anti-inflammatory cytokines IL4 and IL6, variants in which correlate with NS and have a possible role in diagnosis of SRNS (250).

1.4 Genetics and search for disease causing variants

Genetics is a field of science concentrating on the study of genes and inheritability. Its range can vary from molecular, cellular, organism, family and

population to all the way to evolutionary but still the focal point of interest remains in the gene, and in the functional and structural variability of the gene (252). Genetics is classified under biology but combines multiple fields of life sciences and is closely interconnected also with information systems.

The field of study was fathered in 1860s when Gregor Mendel published his study on the inheritance patterns of certain traits in pea plants and their mathematical description (252). Approximately a hundred years later, as a result of the work of numerous researches, it was concluded that DNA was the molecule responsible for the inheritance. However, only after the discovery of the first specific genetic mutations as causative factors underlying inherited diseases, has the field of study begun its remarkable rise in both popularity and significance.

The toolbox of a current day geneticist is a large one, including animal models and variety of functional analyses, but in here I concentrate on the methods most relevant to this thesis, namely sequence analysis and determining disease-causing variants.

1.4.1 Single nucleotide polymorphisms

Typically genomic variants fall into one of five classes: 1) Single nucleotide polymorphisms (SNP) where one nucleotide has been substituted with another, 2) insertions and deletions, where one or more nucleotides have been added or omitted, 3) copy number variation (CMV), where the copy number of a given sequence increases or decreases, 4) inversion, where the given sequence is turned around in position and 5) translocation, where the given sequence is shifted to another position (253). Most abundant of the variants in the human genome is the SNP. There are 10 million SNPs with minor allele frequency higher than 1% making the SNP density approximately one in 300 nucleotides.

Most commonly SNPs lie in the non-coding regions of the genome and they have poorly known effect on the phenotype. When SNPs lie in the coding regions, splice sites or in regulatory regions they may play an essential role in the forming of the varying inheritable traits that make an individual. These traits also include inherited diseases, such as CNF and SRNS.

1.4.2 Linkage analysis and genome wide association analysis

In the latter half of 20th century the genetic linkage analysis was the method of choice for searching regions of the genome linked to studied phenotype, i.e. inherited disease. Linkage analysis is based on the notion that the closer two genetic loci physically are, the more likely they are to be passed on to the next

generation together. The further away two loci are from one another, the more likely it is that recombination occurs between them separating alleles to different copies of a chromosome. Linkage analysis uses a map of genetic markers, short sequences with variable genotype and known physical location in the genome, to find regions where the marker genotypes are linked, inherited together, with the disease phenotype.

Previously genetic markers were microsatellites, few base pairs long sequences repeated 5-50 times, but more recently SNPs have taken their place. While microsatellites are more informative markers, as a single microsatellite can have several different copy numbers in population compared to the typically biallelic SNPs, their frequency in the genome is much lower, and their genotyping is slower and not as readily adaptable to high-throughput analyses (254-257).

When loci are inherited together more often than expected if the loci where independent, they are said to be in linkage disequilibrium (LD). A block of several marker genotypes showing LD is called a haplotype. When a specific haplotype segregates with the disease phenotype, the genes in the genomic region of the haplotype may be considered candidate genes for the disease.

Linkage analysis is especially powerful when searching for candidate genes for rare monogenic diseases in family pedigree data, like for instance in the case of CNF (258). However, when studying complex diseases with common genetic variants of varying, modest effect influencing the phenotype, the method loses power. In these experiments, genome-wide association studies (GWAS) using SNP makers gained popularity. However, the rise of the whole-genome sequencing studies has found new applications for linkage analysis (259).

GWAS is a case-control –study that analyses SNP genotype frequencies in these two populations. If some genotype is significantly more frequent in people with certain phenotype, the two are said to show association. The analysis is carried out using microarrays that contain up to millions of SNPs selected uniformly around the genome. To this date, thousands of GWAS studies have identified tens of thousands SNPs associated with numerous traits and diseases (260, 261).

A challenge with GWASs is that they often result in great number of associated SNPs, most of which are located in the non-coding region, and as the significance of a variant is expected to be modest, it is difficult to study the relevance of the results (262, 263). So far, this seems to be a bottleneck in GWAS protocol in gaining findings of clinical or biological relevance.

1.4.3 Sequencing

Sequencing is a basic molecular biological method to define the order of the nucleotides in a specific DNA strand. Sequencing can be used for instance to determine variants in a gene of interest or in association with a disease phenotype.

1.4.3.1 Sanger Sequencing

The most widely used sequencing method in the past decades has been Sanger sequencing that is based on polymerase chain reaction (PCR), which uses DNA polymerase and free nucleotides to synthesize a new sequence complementary to a template DNA strand originating from a specific primer sequence. The key invention is to substitute a small portion of the nucleotides of the reaction with modified, unextendable, labeled nucleotides (264). This results in a collection of varying length sequences with a labeled nucleotide in the end. The sequences are then arranged by size in an electric field. The sequences float past a detector that records the label, which is used to compile the DNA sequence.

Sanger sequencing is very good method when analyzing limited amount of sequences in a limited amount of samples. It has been often used to as a second step after linkage analysis, to find variants in found genes of interest. However, after the sequencing of the human genome, the interest has shifted towards large scale sequencing experiments, such as whole genome sequencing and whole exome sequencing.

For these Sanger sequencing is not efficient enough. The human genome sequencing project used Sanger sequencing and it took 12 years, cost nearly 3 billion dollars and involved hundreds of researchers around the world (265, National Human Genome Research Institute, https://www.genome.gov/11006943/ (cited 2.6.2016)). Even though its efficiency has advanced since the early days of the Human Genome Project, genome wide Sanger sequencing is not feasible for practical use in most research laboratories or in diagnostic ones. For these applications, next generation sequencing was developed.

1.4.3.2 Next generation sequencing

The term 'next generation sequencing (NGS)' includes several technologies, such as sequencing-by-synthesis (266), sequencing-by-ligation (267) and ion

semiconductor sequencing (268). As the presently predominant technique in the field of medical genetics is sequencing-by-synthesis used by Illumina devices, it is the technique I will concentrate on in this chapter.

Sequencing-by-synthesis method is developed from the Sanger sequencing and share many of its features. This method too uses DNA polymerase synthesis with unextendable, fluorescent nucleotides. A great difference is that in Sequencing-by-synthesis all of the nucleotides are modified, not only a small portion.

The template strand is bound to a specific position on a glass slide. On one glass over 250 million different templates can be bound. The DNA synthesis reaction occurs and one nucleotide is added to the strands to be synthesized. The slide is then photographed with microscopic imaging system and the color and intensity of the fluorescent dyes are registered. Then the newly synthesized nucleotide residues are converted to regular bases, and the slide is ready for the next round of synthesis and imaging. After the last round, information from the collected images is gathered to compile a sequence complementary to the template. All the sequences of a sample are then matched to a reference genome to form a complete read of the genome.

The obvious huge benefit of this technique is the amount of sequences that can be synthesized simultaneously. On this method, the one NGS machine can sequence the human genome in little more than one day (269). Due to its efficiency and cost-effectiveness NGS is in use in clinical laboratories as well as in research. It is used for instance in carrier screening, fetal aneuploidy testing, rare disease detection and assessing the risk and existence of cancer (270-273).

2. Aims of the study

The aim of this thesis was to study the genetic and cellular mechanisms underlying proteinuric kidney diseases steroid sensitive nephrotic syndrome (SSNS), steroid resistant nephrotic syndrome (SRNS) and congenital nephrotic syndrome of the Finnish type (CNF). The specific aims were:

1. To study the predictive value of clinical features and podocyte protein coding gene variants in MCNS

2. To study the association of SNP variants in functional kidney molecules and GC metabolism related genes to the severity of the INS phenotype

3. To study the role of genetic variation in a Finnish cohort of patients with SRNS

4. To study the effects of the absence of nephrin to the expression of key proteins between the SD and the actin cytoskeleton in CNF.

3. Material and methods

3.1 Patients (I, II, III)

The patients involved in this work were diagnosed at the University Hospitals in Finland between 1965 and 1981 (I), or after 1989 (III). Blood samples for genetic analyses were collected during routine check-ups, or on request in their local health centers. The nomenclature of childhood nephrotic syndrome (NS) is confusing due to the overlapping terms. Here, the terminology follows the praxis used in the original articles.

Study I: The study population included 111 MCNS patients. This unselected cohort of patients represents the majority of children with NS diagnosed in Finland at the time. Most patients were enrolled in the International Study of Kidney Disease in Children and were treated by protocols still used in pediatric nephrology. Helsinki University Hospital served as the primary care unit for most of the patients. Long-term data were attained from 104 individuals.

Study II: The study cohort included 100 INS patients of whom 83 were diagnosed between 1965-1981 at the Children's Hospital, Helsinki University Hospital. These 83 were enrolled in the International Study of Kidney Disease in Children and were treated by protocols still used in pediatric nephrology. The remaining 17 patients were diagnosed more recently at the Children's Hospital, Helsinki University Hospital, and were still children or adolescents; of them SNP genotypes were used in statistical comparison between INS patients and controls. A blood sample for DNA extraction was gathered from all.

Study III: Two pediatric siblings with SRNS (Family AM) was the starting point of this study. Seven other family members in three previous generations were recruited. Of these, three were inflicted with SRNS. In addition to Family AM, the cohort included twelve pediatric patients with SRNS. Nine of the twelve patients showed FSGS histology and MCNS. The clinical course of the disease of these patients was variable. Two had neurological symptoms.

3.2 Kidney tissue samples (IV)

The kidney tissue samples came from 20 children with CNF caused by Fin-Major and Fin-Minor mutations. The children underwent nephrectomy at the Children's Hospital, Helsinki University Hospital as a part of the therapy. The nephrectomy was performed at the age of 6-8 months when the renal histology was still well preserved, showing little sclerotic changes. The CNF samples were

analyzed against control samples from 14 adult organ donors whose kidneys were unsuitable for transplantation but still showed normal histology.

The kidney biopsy samples from 11 MCNS patients were collected by core needle biopsies performed on clinical grounds. Patients were divided into two categories, those with proteinuria at the time of the biopsy (n=5) and those in remission at the time of sampling (n=6). Control biopsy samples came from non-proteinuric pediatric patients (n=5) with kidney disorders including IgA nephropathy, Henoch-Schönlein purpura, benign hematuria, and post-infection nephritis.

3.3 Data collection (I, II)

Clinical data from all 104 patients were carefully recorded from the hospital records. The present health status was verified by mailed questionnaires and interviews performed at an outpatient clinic (40 subjects) or by phone (64 subjects). The mean age at the last follow-up was 35.0 years (range 25.1–44.1 years). The questions dealt with possible signs and symptoms of proteinuria or other renal manifestations in adulthood, family history, presence of any other diseases, regular medication, as well as the number of pregnancies and children. Whenever the information obtained from the questionnaire or the first telephone contact was inadequate, the participant was re-interviewed by phone. Also, data were received from the patient's general practitioner or local hospital, when needed. Seventy-seven participants also visited the laboratory at Helsinki University Hospital or their local health centers. They were chosen based on the place of residence (the southern part of Finland) and had different clinical courses and outcomes. The features of the disease (number of episodes, duration of the disease) in these subjects represented well the whole group. Urinalysis was performed by dipstix, and plasma creatinine, albumin and cholesterol were measured in this subgroup.

3.4 Immunostaining (III, IV)

Antibodies used for immunofluorescence staining were goat polyclonal antibodies against ILK (C-19, sc-7516), Integrinα3 (I-19, sc-6592), LMX1β (N-14, sc-21231) and Pod1 (P16, sc-15007), mouse monoclonal antibodies against Nck1/2 (G-12, sc-166425), Smad7 (Z8-B, sc-101152) and ZHX2 (Z-07, sc-101562), and rabbit polyclonal antibodies against Fat1 (HPA023882), Neph1 (H-150, sc-33136), WT1 (c-19, sc-192), ZO-1 (61-7300), podocin (C-18, sc-22296; N-21, sc-22294) and nephrin (UP3).

Antibodies used for immunoperoxidase staining were mouse monoclonal antibodies against α-Actinin-4 (LW-M23, sc-134236), β-dystroglycan (4F7, sc-33702), CD2AP (B-4, sc-25272), Fyn (FYN-59, sc-73388) and Podocalyxin (4F10, sc-23903), rabbit polyclonal antibodies against Nfκb(p50) (NLS, sc-114), N-WASp (H-100, sc-20770) and Pax2 (B24, sc-133889), and goat polyclonal antibody against podocin (N-21, sc-22294).

All antibodies were manufactured by Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA) except antibodies against Fat1 (Sigma-Aldrich Inc., St. Louis, Missouri, USA), ZO-1 (Invitrogen, Carlsbad, California, USA) and nephrin (by courtesy of Karl Tryggvason lab, Karolinska Institute, Stockholm, Sweden).

Immunofluorescence staining of the kidney cryosections (5 μm) was carried out using a traditional protocol. Acetone fixing was applied. Sections used as negative controls were incubated in phosphate-buffered saline (PBS) instead of a primary antibody. Several secondary antibodies were used depending on the primary antibody.

Immunoperoxidase staining was performed on sections of formalin-fixed, paraffin-embedded renal samples using NovoLink Polymer Detection System (RE7150-K, Leica Microsystems GmbH, Wetzlar, Germany) or ImmPRESS Anti-Goat Ig (peroxidase) Polymer Detection Kit (MP-7405, Vector Laboratories Inc., Burlingame, CA, USA). Reaction times were assessed for each antibody by using trial samples and then the same optimized reaction time was used for each patient and control sample to ensure comparable results. For each antibody, all samples were stained at the same time.

3.5 Light microscopy (III, IV)

Light microscopy was performed with a standard Leica DM RX light microscope. Each glomerulus was photographed separately under microscope and the image then imported to Apple Preview 5.0.3 (504.1) application for grayscaling. The semiquantitative scoring was carried out by defining specific staining in each glomerulus and then calculating the area fraction – the portion of specific staining in glomerulus versus background- using NIH ImageJ 1.32j (National Institutes of Health, Bethesda, MD, USA). Between 59 and 121 glomeruli were randomly analyzed in each patient and control sample.

3.6 Genetic analyses (I, II, III)

Genomic DNA was isolated from frozen or fresh peripheral blood samples using the conventional molecular biology technique (Puregene EPTM DNA Purification Kit, Gentra Systems). The analyses of protein coding regions of *NPHS1* (NM_004646), *NPHS2* (NM_014625), *NEPH1* (NM_018240), *CD2AP* (NM_ 012120) and *ACTN4* (NM_004924), as well as exons 2 and 4 of *INF2* (NM_022489) were performed using direct sequencing. Exons were amplified by PCR with flanking intronic primers. The reactions were performed in total volumes of 25 µl as previously described by Lenkkeri *et al*. (316). Occasionally, the denaturation temperature was raised up to 98°C, or betaine was added to the reaction mixture. PCR products were subjected to automated sequence analysis by BigDye-terminator chemistry (v.3.1) on Genetic Analyzer 3730 (Applied Biosystems). The sequences were analyzed with GeneComposer version 1.1.0.1051 (www.GeneComposer.com) or 4Peaks version 1.7.2 (by Griekspoor A and Groothuis T, mekentosj.com.).

When a nucleotide change resulting in an amino acid change was spotted, the significance of the change was studied by sequencing the same exon from 23–51 healthy controls except in the case of the deletion carried by Family AM (study III) which was checked from 101 healthy controls. Also, the healthy members of Family AM were sequenced as controls.

In addition, Blueprint Genetics was commissioned to carry out a Nephrotic Syndrome sequencing panel of nine genes (http://blueprintgenetics.com/nephrotic-syndrome-panel/) to four healthy and three affected members of Family AM. While the panel included *NPHS1, NPHS2, ACTN4* and *TRPC6* that were previously sequenced it also contained all the rest of the *INF2* exons as well as genes *CD2AP, LAMB2, PLCE1* and *WT1*. The panel targets all exons, intron/exon boundaries and known mutations outside these regions.

The effects of amino acid changes were evaluated by using online bioinformatics tools Predict Protein server, Prosite database and PolyPhen.

Whole genome sequencing (WGS) method was used to analyze the sequences of the coding and non-coding areas of *NPHS2*, *NPHS1*, *INF2*, *CD2AP*, *WT1, TRPC6, ACTN4* and *PLC1*. The experiment was carried out in two affected (II-1 and IV-2) and two healthy (I-1 and II-2) members of the family AM in the Finnish Institute of Molecular Medicine (www.fimm.fi, University of Helsinki, Biomedicum, Helsinki Finland). The whole genome sequencing data analysis was conducted using Integrative Genome Viewer (www.broadinstitute.org/igv/).

3.7 Single nucleotide polymorphisms (II)

We selected eleven SNPs from eight genes to this study based on previous reports in literature on their association with INS. The genes and SNPs were *Angptl4* SNP rs1044250 (c.797C>T), *GPC5* SNP rs16946160 (c.325+102637G>A), *IL-13* SNP rs848 (c.*526C>A), *MIF* rs755622 (c.–270G>C), *nNOS* rs2682826 (c.*276C>T), *MDR1* SNPs rs1128503 (c.1236C>T), rs2032582 (c.2677G>T/A), rs1045642 (c.3435C>T), *GLCC11* SNPs rs37972 (c.-1473C>T) and rs37973 (c.-1106A>G) and *NR3C1* rs41423247 (c.1184+646G>C).

3.8 Plasmids (III)

Wild type (WT) and c.988_989delCT -mutated NPHS2 sequences were inserted to pCMV-Myc –vector (Clontech, Palo Alto, CA, USA) to give them N-terminal Myc-tag. The WT-NPHS1 was inserted to pcDNA3.1 –vector (Invitrogen, Carlsbad, CA, USA). Plasmids were amplified using JM109 competent E.coli cells, QIAGEN Plasmid Maxi Kit (QIAGEN inc. Valencia, CA, USA) and the protocol provided by the kit.

3.9 Co-immunoprecipitation of nephrin and podocin (III)

HEK293FT cells were transiently transfected with two plasmids: one carrying WT-nephrin, the other carrying either WT-podocin or c.988_989delCT -mutated podocin. The transfection was carried out using FuGENE 6 –protocol (Roche, Basel, Switzerland). After incubating for 24 hours the cells were washed in PBS and lysed in non-denaturing lysis buffer containing 20 mM TrisHCl pH8, 139 mM NaCl, 10% glycerol, 1% NP40, 2 mM EDTA, and phosphatase and proteinase inhibitors. Cell lysates were incubated with c-Myc (A-14) antibody (Santa Cruz Biotechonology Inc., Santa Cruz, CA, USA) over night at +4°C on gentle agitation. After this, 50 μl 50% rec Protein G-Sepharose beads (Invitrogen, Carlsbad, CA, USA) were added to the antibody-protein complex mix and incubated over night at +4°C on gentle agitation to bind the antibody to the beads. Beads were washed with the lysis buffer and fractioned by SDS-PAGE. Western blot analysis was performed using nephrin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by the incubation with horseradish peroxidase-coupled anti-goat antibody. Immobilized antibodies were detected by using chemiluminescence.

3.10 Data analysis and statistics (I, II, IV)

The statistical examination of the data was carried out with statistical software JMP 9 and 10 (www.jmp.com, SAS Institute Inc., Cary, NC, USA).

Study I: Logistic regression analysis was used to calculate odds ratios (OD) and 95% confidence intervals (CI) for the effects of each predictor variable on these outcomes. T-test was used to compare background clinical data between groups (Mann Whitney in the case of non-normally distributed variable).

Study II: Logistic regression analysis was used to calculate odds ratios and 95% CI for the association between genotypes and the risk of INS. The difference between selected clinical variables and genotypes was determined by Fisher's exact test.

Study IV: Data are presented as a median with interquartile range (IQR). The differences between control and CNF patients were evaluated using Mann-Whitney-Wilcoxon test. All figures were composed using Adobe Photoshop CS3 imaging software (http://www.photoshop.com/).

P-values < 0.05 were considered significant.

3.11 Ethics (I, II, III, IV)

All patients or their parents were informed of the content of the study and they signed a form of consent. The study was approved by the Ethics Committee of the Children's Hospital, Helsinki University Hospital.

4. Results

4.1 Clinical characteristics of childhood MCNS (I)

4.1.1 Clinical features of childhood MCNS

The clinical features of the 104 analysed patients are summarized in Table 2. Twenty-three patients had only one episode of nephrotic range proteinuria while a majority of the patients relapsed during childhood (81 patients, 78%). The amount of relapses showed considerable variation (range 1–28 relapses, median 3). Fourteen patients (13.5%) continued to suffer from proteinuria in adulthood. Three of them had occasional mild proteinuria while three had condition severe enough to cause constant proteinuria and were under continuous corticosteroid medication. Eight of the fourteen had a mean number of 1.9 nephrotic episodes in adulthood (range 1–4 years). The period between the childhood and adulthood episodes varied (range 2-17 years) and the mean interval between episodes in adulthood was 4.6 years (range 1–11 years). None of the patients developed renal failure.

4.1.2 Long-term outcome of childhood MCNS

The interviews revealed that the general health of the participants was good in adulthood. None had lymphomas or other types of cancer. One participant was treated for hypercholesterolemia. Seventeen percent reported some allergic problems, mainly hay fever, which is in accordance with their prevalence in the Finnish population. The prevalence of diabetes (4.2%), asthma (6.7%) and ulcerative colitis (2.9%) was slightly elevated from the prevalence in the Finns of similar age (the respective figures: 2.5%, 4.1% and 0.4%; statistics of the National Public Health Institute, Helsinki, Finland). One participant had both diabetes and ulcerative colitis and one had a combination of diabetes, ulcerative colitis and psoriasis.

4.1.3 Clinical Predictors in MCNS

Severe course of the disease was not associated with analysed features such as mesangial hypercellularity in kidney biopsy, male sex, and young age of onset. No association between autoimmune disease in adult life and severity of childhood MCNS was observed. However, subjects with adulthood proteinuria had a tendency for a complicated disease already in childhood. These complications included a higher number of childhood episodes (mean 15.6 vs.

4.9 episodes, OR for each additional episode 1.19 (95% CI 1.10 to 1.30), P <0.0001), more frequent steroid dependency (57% vs. 10%, OR 12.0 (3.4 to 42.4), P =0.0001) and need for cytostatic drugs more often (71% vs. 35%, OR 3.9 (1.1 to 13.5), P =0.03) as compared to those with episodes only in childhood. None of the parents, siblings or offspring of the study subjects had nephrotic kidney disease. Temporary proteinuria was reported in first-degree relatives of four participants.

Table 2 The clinical characteristics of the kidney disease in the study cohort

Characteristics	**Patients** N=104
Males (%)	74 (71)
Age and Duration (mean (range))	
Age at last follow-up in years	35.0 (25.1-44.1)
Follow-up time in years	30.0 (20.8-37.9)
Age at onset of MCNS in years	5.0 (0.65-13.1)
Duration of the disease in years	4.6 (0.1-33.4)
Biopsy (%) (n=86)	
No/minimal changes	69 (80)
Mesangial hypercellularity	17 (20)
Childhood episodes (%)	
One episode	23 (22)
2-5 episodes	46 (44)
6-10 episodes	16 (15)
>10 episodes	19 (18)
Frequent relapses	54 (52)
Adulthood episodes (%)	
Nephrotic episodes	11 (11)
Temporary proteinuria	3 (2.9)
Medication (%)	
Steroid dependency	17 (16)
Cytotoxic therapy	45 (43)
Steroid treatment at the last follow-up	5 (4.8)

4.2 Genetic variation in INS (I, II, III)

4.2.1 Variants in podocyte protein coding genes in MCNS

No homozygous missense or nonsense variants were found in the direct sequencing of genes coding for the four slit diaphragm proteins nephrin, podocin, Neph1 and CD2AP of the 38 MCNS patients. *CD2AP* was highly

conserved, and not even synonymous changes were uncovered. Similarly, *NEPH1* had no amino acid changing variants, and only three synonymous substitutions in exons 4, 8 and 13. In the *NPHS2* gene p.E87K variant was found in one patient, and not in the controls. Heterozygous variant leading to p.R229Q was detected in four MCNS patients (11%) and in five of the 51 controls (10%). In addition, five synonymous substitutions were found in the *NPHS2* gene both in the study subjects and in controls.

NPHS1 had more genetic variation. Seven out of the 38 patients (18%) had nine heterozygous variants leading to amino acid changes. None of these were found in controls. Five synonymous substitutions were detected both in MCNS patients and in controls.

The analysis included three patients with proteinuria in the first degree relative. One patient, whose father and brother had proteinuria but no nephrotic episodes, had p.T291 amino acid substitution in nephrin and p.R229Q substitution in podocin. The second patient, whose mother had temporary proteinuria, had heterozygous p.R800C in nephrin. The third patient, whose sister had temporary proteinuria, had only known benign polymorphic changes in nephrin and podocin genes.

Also, the analysis included nine subjects with adulthood proteinuria and three of them had mutations in nephrin or podocin genes. One had the Fin-major deletion and a missense mutation leading to p.R800C in the nephrin gene. His proteinuria in adulthood was temporary without clinical manifestations. In the laboratory control, his plasma creatinine level was little elevated (1.43 mg/dl; GFR 80 ml/min/1.73m^2). No biopsy findings were available. The second patient had a single amino acid change in nephrin (p.S1058L). The third patient had two amino acid changes in nephrin (p.S786N and p.G879R) and 1 substitution in podocin (p.E87K). Table 3 summarizes the found amino acid changing variants and the clinical features of their carriers.

4.2.2 Variants in podocyte protein coding genes in Finnish pediatric SRNS

We carried out direct sequencing exons and exon/intron boundaries of *NPHS1, NPHS2* and *NEPH1* in a cohort of Finnish pediatric patients with sporadic SRNS. This analysis showed very little variation. One of the twelve sporadic SRNS cases in the study had an amino acid changing variants in *NPHS2*, p.P341R in one allele and p.R229Q in two alleles. SRNS was diagnosed in this patient at the age of six and she is now waiting for the kidney transplant at the age of 19 years. In the *NPHS1* gene, six of the twelve patients had one or more of three known amino acid changing polymorphisms (p.E117K, p.R402Q, p.N1077S), which were also displayed in the healthy controls in similar

frequencies. No patient carried amino acid changing variants in the *NEPH1* gene. A few synonymous substitutions were detected in all three genes.

Table 3 The number of *NPHS1* and *NPHS2* variants leading to amino acid substitution and clinical manifestations of the patients carrying them.

Subjects with <5 relapses in childhood	**n=12**	
No Changes	8	
Amino Acid substitutions:		
NPHS1	2	1 patient with a NPHS1 and NPHS2 variants
NPHS2	3	
Subjects with 5-16 relapses in childhood	**n=6**	
No Changes	5	
Amino Acid substitutions:		
NPHS1	1	
NPHS2	0	
Subjects with slow response to GC treatment or GC dependency	**n=11**	
No Changes	9	
Amino Acid substitutions:		
NPHS1	1	2 variants in one subject
NPHS2	1	
Subjects with proteinuria in adulthood	**n=9**	
No Changes	6	
Amino Acid substitutions:		
NPHS1	3	2/3 subjects had two variants
NPHS2	1	

4.2.3 SNPs in INS

We compared the frequencies of eleven SNPs in eight genes between INS patients and controls, as well as between subgroups of INS patients.

The clinical variables in the analysis included: age of onset (< 3 yr vs. > 3 yr), number of relapses (<5 vs. >5), frequent relapses (no vs. yes), response to glucocorticoids (GC) (normal vs. slow/no response), and treatment (only GC vs. GC together with immunosuppressive drugs (IS)). Notably, the majority of the patients included to this study were steroid sensitive (response to GC 84 vs. 16%).

4.2.3.1 Comparison between patients and controls

Five SNPs from five genes encoding for functional kidney molecules and six SNPs from three genes involved in steroid metabolism were analysed from 100 INS patients. The distribution of observed genotypes was consistent with those expected under the assumptions of the Hardy-Weinberg Equilibrium (p>0.05).

No association between variant frequencies and disease status was observed except in rs848 variant in *IL-13* gene, where patients and controls differed in the frequency of the CA genotype (53% vs. 38%, OR 2.025, CI 1.095-3.785, p=0.0243).

Table 4 The associations of SNPs in functional kidney genes and their comparison to recent studies.

Gene	Found association	Current study	Referenced study
Angptl4	rs1044250 (c.797 C>T)		
	Association with C allele and IS medication need		
GPC5	**rs16946160 (c.325+1026376G>A)**		
	Association of AA genotype with NS	No	Yes[1]
	Association of A allele with disease onset	Yes	
	A allele frequency	0.168	0.08[1]
IL13	**rs848 (c.*526C>A)**		
	Association of genotype distribution with long-term outcome	No	Yes[2]
MIF	**rs755622 (-270G>C)**		
	Association of C allele and/or GC genotype with NS	No	Yes[3]. Yes[4]. No[5]
	Association of CC genotype with GC resistance	No	Yes[3]. Yes[4]. No[22]
nNOS	**rs2662826 (c.*276C>T)**		
	Association of TT genotype with NS	No	Yes[6]
	Association of TT genotype with GC responsiveness	No	No[6]

[1] Okamoto *et al.* 2011 (175) [2] Wei *et al.* 2005 (274) [3] Berdeli *et al.* 2015 (239)
[4] Vivarelli *et al.* 2008 (240) [5] Choi *et al.* 2011 (249) [6] Alasehirli *et al.* 2009 (275)

4.2.3.2 Functional kidney molecules

The SNPs in the genes *Angptl4, GPC5, MIF, nNOS* and *IL-13* encoding the functional kidney proteins revealed few statistically significant correlations to the clinical variables. *IL13, MIF* and *nNOS* showed none. In *Angptl4* SNP rs1044250 the C allele was more frequent in patients who received other IS drug medication instead of GCs only (72 vs. 54%, p=0,0228). In GPC5, we observed an association between rs16946160 A allele and early disease onset (16 vs. 5%,

p=0,0421). The findings and their comparison to recent studies can be found in Table 4.

4.2.3.3 GC metabolism related molecules

Analysed genes included *MDR1, NR3C1 GLCCl1*. The genotype distribution in *MDR1* SNPs showed difference in patients who received only GCs compared to those who also received other IS medication as allele T frequency was higher in the latter group in rs1128503 (70.6 vs. 28.6%, p= 0.0012), in rs2032582 (67.9 vs. 42.9%, p= 0.0028) and in rs1045642 (73.1 vs, 52.4%, p= 0.0092).

A curious finding was that *NR3C1* SNP rs41423247 heterozygous GC genotype was more frequent in patients with more than five relapses (68 vs. 32.7%) and in patients with frequent relapses (60 vs. 34%) as well as in patients with severe course of the disease compared to those with milder course (63 vs. 34%).

In *GLCCI1* SNP rs37973 A allele was more frequent in patients who received other IS medication (67 vs. 50%, p= 0.0387). Patients with more than five relapses also had *GLCCI1* SNP rs37973 A allele more frequently than those with fewer than five relapses (70 vs. 52%, p= 0.0377).

The results and their comparison to recent studies can be found in Table 5.

4.2.3.4 Haplotype analysis of MDR1 SNPs

Summary of the haplotype analysis of the MDR1 variants rs1236, rs2677 and rs3435 can be seen in Table 6. The analysis revealed twelve estimated haplotypes in cases and controls. In patient samples the allele frequency of the two most common haplotypes, TTT and CGC, was 70% (42.2 and 27.5% respectively) while in the control samples it took four most common haplotypes, TTT, CGC, CTT, and TGT, to achieve the same (23.4, 18.6, 15.6 and 12.6% respectively).

The distribution of the allele frequencies of the haplotypes showed significant difference between patients with less than five relapses and patients with more than five relapses (haplotype CTC frequencies 40.6 vs. 55.3%, respectively, p= 0.04). In other variants, no statistically significant variation can be seen. In all variants, TTT and CGC haplotypes had the combined allele frequency varying between 71 and 86.7%, and no other haplotype had allele frequency above 10%.

Table 5 The associations of SNPs in GC metabolism related genes and their comparison to recent studies.

Gene	Found association	Current study	Referenced study
MDR1	**rs1128503 (c.1236C>T)**		
	Association of T allele and/or TT genotypes with IS medication need	Yes	
	rs2032582 (c.2677G>T/A)		
	Association of genotype distribution with NS	No	No[1]. Yes[2]. Yes[3]. No[4]
	Association of CC genotype with age of onset	No	No[1]. No[2]. Yes[3]. No[4]
	Association of T allele and/or TT genotype with GC responsiveness	Yes	
	Association of T allelle and/or TT genotypes with IS medication need	Yes	
	rs1045642 (c.3435C>T)		
	Association of T allele and/or TT genotype with NS	No	Yes[1]. Yes[2]. Yes[3]. No[4]
	Allele frequencies of controls (C/T. %)	37.2/62.8	45.2/54.8[1]. 66.4/33.6[2]. 58.5/41.5[3]. 42.0/58.0[4]. 42.4/57.6[5]
	Association of CC genotype with age of onset	Yes	No[1]. No[2]. Yes[3]. No[4]
	Association of T allelle and/or TT genotypes with IS medication need	Yes	
	Haplotype		
	Association with GC responsiveness	No	Yes[1]. Yes[3]. Yes[4]. No[5]
	Frequency of TGC haplotype (Case/Control. %)	4.2/7.8	1.1*[1].8.3/6.5[2]. 21.7/18.6[3]. 21.2/18[4]. 1.1/2.3[5]
GLCCI1	**rs37972 (c.-1473T>C)**		
	Association of genotype distribution with GC responsiveness	No	No[6]
	rs37973 (c.-1106G>A)		
	Association of genotype distribution with GC responsiveness	No	No[6]
	Association of A allele with patients with more than five relapses	Yes	
	Association of A allele with IS medication need	Yes	

[1] Wasilewska *et al*. 2007 (248)
[2] Jafar *et al*. 2011 (250)
[3] Youssef *et al*. 2013 (276)
[4] Choi *et al*. 2011 (249)
[5] Cizmarikova *et al*. 2015 (277)
[6] Cheong *et al*. 2012 (245)
*Only control

Table 6 Most common estimated haplotypes in INS patients vs. controls and in patients with <5 relapses and >5 relapses.

Haplotype	Patients (n=82) n (%)	Controls (n=98) n (%)	OR (95% CI)	*p*
TTT	69 (42.2)	46 (23.4)	0.42 (0.27-0.66)	***0.002****
CGC	45 (27.5)	36 (18.6)	0.60 (0.36-0.98)	***0.0412****
CTT	13 (8.1)	31 (15.6)	2.18 (1.10-4.33)	***0.0254****
TGT	7 (4.5)	25 (12.6)	3.28 (1.38-7.79)	***0.0072****
CGT	11 (6.5)	20 (10)	1.58 (0.73-3.40)	*0.242*
TGC	7 (4.2)	15 (7.8)	1.86 (0.74-4.67)	*0.1877*
CTC	2 (1.3)	15 (7.7)	6.71 (1.51-29.80)	***0.0123****
Other	9 (5.7)	8 (4.3)	0.73 (0.28-1.94)	*0.5425*

Haplotype	Relapses <5 (n=52) N (%)	Relapses >5 (n=25) N (%)	OR (95% CI)	*p*
TTT	42 (40.4)	28 (55.3)	1.88 (0.95-3.71)	***0.04****
CGC	32 (30.8)	16 (31.4)	1.06 (0.51-2.91)	*0.88*
Other	30 (28.8)	6 (12)	0.19 (0.02-1.54)	*0.1205*

4.3 Podocyte protein coding genes in Finnish familial SRNS (III)

4.3.1 Rare clinical course of familial SRNS

The first member of Family AM with NS is a daughter of healthy parents (II-1 in Figure 7). She was diagnosed by chance with proteinuria at the age of 18 in a routine check-up. As at that time she was symptomless it is impossible to say how long the proteinuria had continued. A kidney biopsy showed only minimal changes. However, a new biopsy taken seven years later when the patient was 25 years old showed the tissue damage had progressed to FSGS. By this time the proteinuria had worsened significantly. The patient reached ESRD at the age of 40 and received a kidney transplant a year later. The disease has not recurred after transplantation.

The patient II-1 had two sons, both of whom were diagnosed with NS at young age (2,5 (III-2) and 1,8 years (III-3)). Both sons had only low level of proteinuria as a child but it worsened dramatically by the time they were young adults. The elder of the boys developed ESRD at the age of 25 and received a kidney

transplant at the age of 31. The younger son has not yet reached ESRD but in his latest check-up his kidney function had severely worsened.

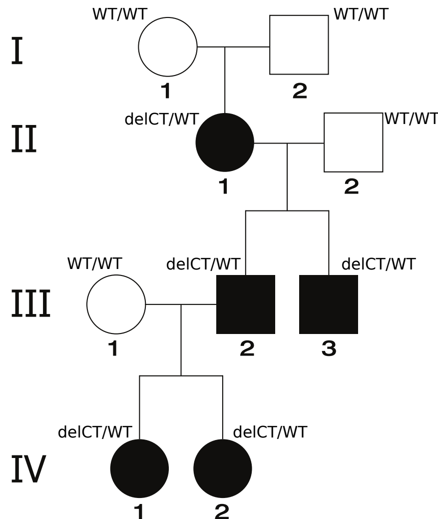

Figure 7 The family tree of Family AM. Filled symbols denote family members with SRNS

The elder son has two daughters, both having proteinuria diagnosed soon after birth (IV-1 and IV-2). The elder of two is 12 years old and at the latest check-up her proteinuria had worsened but is still under 4 g/l. The younger is 3,5 years old and her proteinuria is still under 1 g/l.

4.3.2 NPHS2 in the familial NS

The gene sequence of *NPHS2* was analysed in several ways. It was analysed using direct sequencing of exons and exon/intron boundaries, it was included to the NS gene panel that checked relevant intronic regions in addition to exon and exon/intron boundaries, and was thoroughly checked during WGS analysis that included all the intronic and control regions.

Only one variant was found in *NPHS2* that was carried by only the affected patients in the family and none of their healthy family members or unrelated controls. Neither was any combination of variants found that would have separated the affected from the healthy.

The variant that was found, a heterozygous two nucleotide deletion c.988_989delCT (Figure 8), is a *de novo* variant of the first affected member of the family (II-1) as the patient's healthy parents do not carry the variant. Her two affected sons and the two affected daughters of the eldest son all inherited the variant.

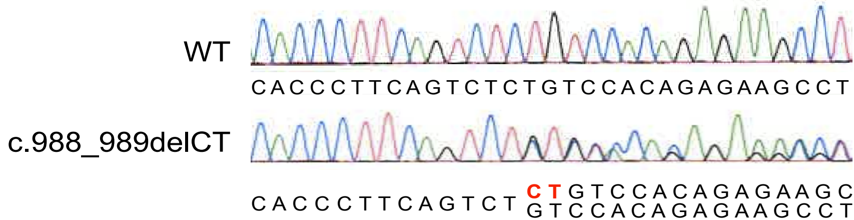

Figure 8 The deletion c.988_989delCT of the Family AM.

The c.988_989delCT affects the last 52 amino acids of the 384 amino acid protein podocin; amino acid 329 is followed by frame shift and premature stop codon 14 amino acid later. Thus, the *NPHS* with c.988_989delCT*2* codes for p.L329fs*14 and leads to podocin protein that only has 343 amino acids.

We studied the effects of c.988_989delCT to the function podocin and its expression in the kidney tissue. Immunofluorescence staining showed that while the patients in Family AM still expressed nephrin and podocin in their podocytes, the staining of nephrin was qualitatively altered and discontinuous, and the staining of podocin was much diminished (Figure 9A). Co-immunoprecipitation experiment showed that truncated podocin binds to nephrin similarly to wild type podocin (Figure 9B). The Predict Protein revealed that the two- and three-dimensional structure of the podocin was not drastically altered by c.988_989delCT. Nine proline residues are lost by the truncation.

4.3.3 Other podocyte genes in familial SRNS

Possible variants causing the NS in Family AM were also searched for in other genes coding for podocyte proteins. We included genes variants in which are associated with dominantly inherited SRNS (*ACTN4, INF2, TRPC6, WT1* and *CD2AP*) as well as genes associated with recessive SRNS and NS in mouse models (NPHS1, *LAMB2*, *PLCε1* and NEPH1).

Analysis of *NPHS1, NEPH1*, and *ACTN4* using direct sequencing revealed only known polymorphisms. The nephrotic syndrome panel of Blueprint Genetics analysed genes *CD2AP, WT1, LAMB2, TRPC6*, *PLCε1 and INF2* in addition to *NHS2* and *NPHS1*, and did not find any variant in any gene that would have followed the disease phenotype. The WGS revealed one variant in SRNS

associated gene that was shared by the two affected family members included to the WGS analysis (II-1 and IV-2) but not the two healthy ones. Direct sequencing of this non-coding variant (a single nucleotide deletion (rs76500597) in *WT)* in the rest of the family members revealed no shared genotype between the affected individuals: one affected did not carry the deletion while two healthy family members did (one as a homozygote).

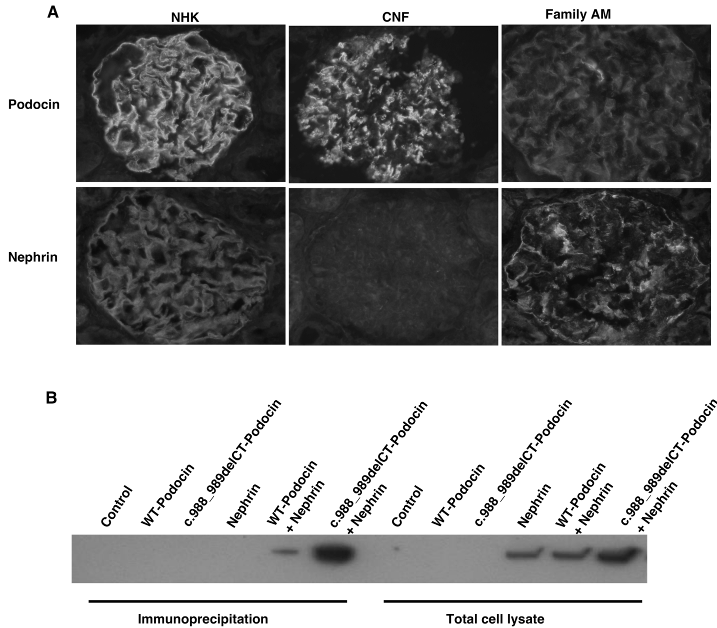

Figure 9 Functional study of c.988_989delCT. **A** Expression of nephrin and podocin in normal human kidney (NHK), CNF and Family AM kidney. The biopsy section came from patient IV-1. 40x magnification. **B** Co-immunoprecipitation of WT-podocin and c.988_989delCT-podocin with nephrin.

4.4 Podocyte Proteins in kidney disease (IV)

The expression of several proteins from different cellular compartments of podocytes in CNF was stained and analysed using Immunohistochemistry. The main findings are presented in Table 7 and in Figure 10. The expression of few key proteins were analysed from MCNS kidney.

4.4.1 Podocyte proteins in CNF

The expression of slit diaphragm proteins in the analysis was reduced in CNF glomeruli. While the Fat1 expression showed only moderate decrease (2,6-fold in area fraction) the expression of Neph1 was drastically diminished (45-fold).

The expression of adaptor proteins was close to controls in the case of podocin and NCK1/2 (1,1-fold and 1,4.fold increase, respectively), and moderately increased in the case of CD2AP (3,3-fold).

Actin cytoskeleton proteins ACTN4 and INF2 showed mildly increased staining (2.2-fold and 1.7-fold, respectively) while N-WASp staining was not significantly altered.

The staining for basal membrane domain protein α3-integrin showed a small increase (1,9-fold) in CNF. On the other hand, the staining of the other basal membrane domain protein in the analysis, β-dystroglycan, was reduced (2,6-fold). The only protein of the apical membrane domain included to the analysis was podocalyxin, staining of which showed slight decrease (1,2-fold).

Table 7 CNF staining in CNF. This table collects the main findings of the study. NHK, normal human kidney.

Proteins	Number of glomeruli		Median (IQR)		Regulation (fold change)
	NHK	*CNF*	*NHK*	*CNF*	
Neph1	83	83	6,4 (7,9)	0,1 (0,2)	down (45,8)
Fat1	84	84	1,6 (0,6)	0,6 (0,7)	down (2,6)
CD2AP	110	121	1,8 (3,2)	5,9 (3,9)	up (3,3)
ACTN4	89	108	1,4 (2,9)	2,9 (2,7)	up (2,16)
β-Dystroglycan	103	114	7,0 (2,7)	2,7 (4,0)	down (2,6)

4.4.2 Podocyte proteins in MCNS

The staining of Neph1, FAT1, CD2AP, and ACTN4 was studied in the MCNS biopsy samples taken during proteinuria and in remission. In contrast to CNF kidneys, the staining of Neph1 was only marginally reduced (1.5- and 1.6-fold in MCNS with and without proteinuria, respectively), as was the staining of ACTN4 (1.5- and 1.6-fold in MCNS with and without proteinuria, respectively). Neither CD2AP nor FAT1 showed any significant changes in staining.

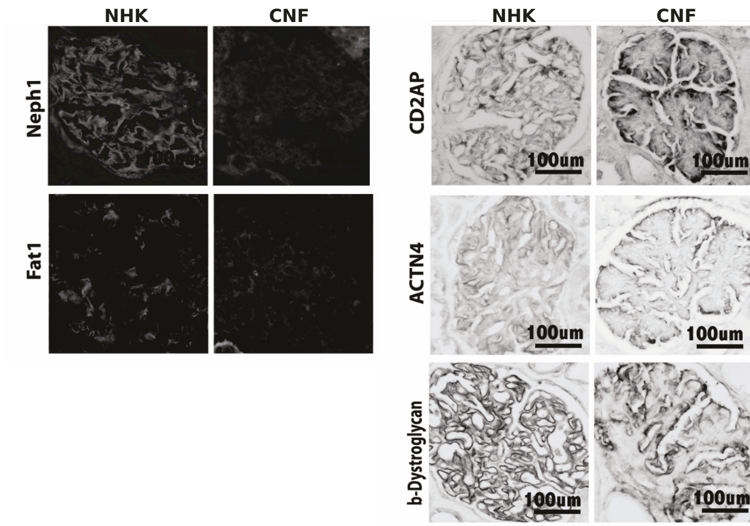

Figure 10 The staining of key podocyte proteins in CNF kidney section. 40x magnification

5. Discussion

5.1 Clinical characteristics of childhood MCNS (I)

MCNS is the most common form of NS in children. It is a complex disorder with still elusive pathogenesis. It is usually associated with steroid sensitivity. While clear cut causative variants are not likely to have a major, wide-spread role in MCNS, the question if genetic variation has a minor or modifying effect on the disease phenotype is still very much an open one. As the course and severity of MCNS cannot be assessed by a simple DNA test it would be highly beneficial to find other predictors with prognostic value, such as demographic and clinical features of the patients. We mapped long-term outcome and clinical features of 104 Finnish adults with history of childhood MCNS to define reliable risk factors. We also looked into the overall morbidity of the MCNS later in life.

5.1.1 Clinical features and long-term outcome of childhood MCNS

Based on our results a child at their first occurrence of MCNS has approximately a 20% chance of no relapses, a 65% chance of relapses limited to childhood and a 15% chance that the proteinuric episodes still occur in adulthood. The median expected number of relapses is three and the duration of the disease is five years. If the child becomes dependent of steroids or has more than ten episodes in childhood, the chance of proteinuric episodes in adulthood increases to over 40%. A study by Skrzypczyk *et al.* (278) show similar results as patients with relapses in adulthood have mean value of ten relapses in childhood compared to the mean value of four relapses of the patients with childhood only condition. Skrzypczyk *et al.* also observed that steroid-dependency in childhood was more frequent in patients with relapses in adulthood but due to small patient number the difference was not statistically significant.

Over all, the outcome in MCNS appears to be good. Previous studies with shorter follow-up times have reached this conclusion (218, 219, 278-280). The studies report that 5-19% of the patients had proteinuric episodes as adults, with the exception of Fakhouri *et al.* (280) who reported adulthood episodes in 42% of the patients. This high percentage may be affected by their selection of patients who are mostly steroid dependent.

Several studies have reported an association between MCNS and immunological disorders (asthma, allergy and autoimmune diseases) (223, 281). Our study is in concordance with these previous reports: the patients may have some—although not strong—tendency to autoimmune disorders. Both MCNS and in allergic

disorders show activation of Th2 helper cells and cytokines such as IL4 and IL13 (282). In MCNS proteinuric episodes often start during viral infection and it is possible that immune mediators play a significant role in it. Abdel-Hafez *et al.* (283) suggest that there is no causal relationship between allergic disorders and MCNS but underlying immunological events lead to both conditions. In particular IL13 may mediate proteinuria in MCNS patients as it induces the expression of protein CD80 in the podocytes, which has been associated with proteinuria. However, as the association of immunological disorders and MCNS is so weak in our study, it may indicate that the extent of these mechanisms is limited.

5.1.2 Clinical Predictors in MCNS

MCNS it does not run in families as clearly as SRNS is known to do. Thus, family history is not a comprehensive predictor of SSNS. However, there have been studies reporting familial SSNS in different parts of the world (284-286). Most of these incidences involve patients from consanguineous families that explain the probable accumulation of various genetic factors. Recently Gee *et al.* (160) reported two consanguineous Turkish families with monogenic cause for SSNS, homozygous variants in *EMP2* gene. Consanguinity, however, is not always the case. Ruf *et al.*, (284) carried out a linkage analysis and pinpointed SSNS causing locus (SSNS1) on chromosome 2p in one family. However, since then the genes of this locus have not been associated to familial SSNS in larger scale. Genetic heterogeneity seems to be explicit in MCNS inheritance and mapping out the family history and especially the known consanguineous marriages in the family may provide relevant information.

5.2 Genetic variation in INS (I, II, III)

The genetic background of majority of INS cases is still undiscovered. Most findings concern SRNS cases, which have revealed causative variants in podocyte protein coding genes but this subset is only approximately 30% of all reported cases and majority is still undefined. In SSNS causative genetic variants are rare. This is not unexpected as SSNS generally is considered a complex trait. Genetic variants may however play a minor or modifying role in the disease phenotype. The best predictor of the severity of the disease is the patient’s response to steroids but even that is not always reliable and initial response may change in time. Prolonged GC medication carries the risk of severe side effects including obesity, Cushing syndrome, hypertension, bone disease, growth retardation, striae, cataracts and behavioral disturbances (287). If patient’s response to GC medication could be reliably predicted at the time of diagnosis, the patients could get suitable treatment faster and avoid useless damaging medication. We looked into podocyte protein coding genes in MCNS

and SRNS patients and known variants in genes coding for other functional kidney molecules and proteins involved in steroid metabolism in INS patients to find out their usefulness in predicting the disease phenotype.

5.2.1 Variants in podocyte protein coding genes in MCNS

An attractive hypothesis is that the protein leakage in MCNS is caused by a combination of structural weaknesses and transient, circumstantial factors, such as T-cell cytokines. As the podocyte foot processes and the SD play an essential role in protein retention and are found to be damaged in certain other forms of NS, genes coding for proteins involved in this cellular structure were of interest to us.

We carried out direct sequencing of exons and exon/intron boundaries of *NPHS1*, *NPHS2* and *NEPH1*. Few variants in these essential podocyte protein-coding genes were found, which leads to the conclusion that their meaning to the development of the MCNS condition is limited and minor. Other studies have reached similar conclusion. Kyrieleis *et al.* (288) found nothing but three heterozygous variants in *NPHS1,* and in *NPHS2* only p.R229Q as a heterozygote. The other genes in their study (*CD2AP* and *ACTN4*) revealed no variants. Gbadegesin *et al.* (289) analysed *NPHS2* and *WT1* patients with steroid dependant or frequently relapsing NS and found no variants.

Interestingly, in our study more variants accumulated to MCNS patients than to controls in *NPHS1*, but the pathogenic or modifying role of the variants is unclear and as the difference between patients and controls was small, little can be said about their significance to the MCNS phenotype in this population.

5.2.2 Podocyte protein coding genes in the Finnish pediatric patients with SRNS

Internationally, SRNS patients with determined causative variants predominantly present with histological finding FSGS (185). In the Finnish population FSGS is rarer than in other populations in general (2 vs. 4-8%, (218)). The reason for this is not known. We were curious if the frequency and nature of genetic variants in podocyte protein genes in Finland were comparable to those found elsewhere in the world or if the there was something in the Finnish genetic makeup that could explain the difference in prevalence.

Few variants were discovered in *NPHS1, NPHS2* and *NEPH1*. Most notably one of the twelve sporadic SRNS cases in the study had an amino acid changing variants in *NPHS2*, p.P341R in one allele and p.R229Q in two alleles. SRNS was diagnosed in this patient at the age of six and she is now waiting for the kidney

transplant at the age of 19 years. p.R229Q is a curious variant, pathogenicity of which have been contemplated during the years. It is associated with late-onset SRNS and albuminuria in general population but is also observed as a homozygote in healthy controls (290, 291) Recently, Tory *et al.* (190) suggested that it is only pathogenic together with another disease causing *NPHS2* variant (compound heterozygosity), and that its pathogenicity is dependent on the position of the other variant. As p.P341R variant is in exon 8 this finding is in accordance with this proposed model.

While Neph1 is associated with NS only in animal models (93) and *NPHS1* variants are predominantly found in congenital NS, the frequency of disease-causing *NPHS2* variants in sporadic SRNS is reported to be 10-28% (179, 180, 292). In the cohort included in this study there was only one patient with potentially disease causing variants in the gene. This is slightly less than anticipated based on literature (8,3% vs. 10-28%). Frequencies of disease-causing variants in the genes behind SRNS are known to differ between populations (293, 294). FSGS is relatively rare in Finland and it is possible that lack of *NPHS2* variation is the reason behind this. However, the cohort size in this study is insufficient to draw conclusions, and larger scale study must be conducted in order to clarify the matter.

5.2.3 Single nucleotide polymorphisms in INS

We genotyped eleven SNPs from eight genes (*nNOS, IL-12, MIF, Angptl4, GPC5, MDR1, NR3C1* and *GLCCI1*), which had previously been studied in relation to INS and proteinuric animal models. No great differences were observed between patients and controls, and all in all the observed associations between SNPs and clinical features were modest.

5.2.3.1 The functional kidney molecules

Alasehirli *et al.* (275) found that in *nNOS* gene polymorphism rs2682826 the TT genotype was associated with INS but not with GC responsiveness. NO attenuates many functions in the kidney and all forms of *NOS* are expressed in the kidney but the role of NO in renal disease is unclear. We did not see any association of rs2682826 genotypes to INS nor to any clinical features of the disease.

Our results of the two cytokines, *IL-13* and *MIF* were also negative. While Wei *et al.* (274) reported that 3'UTR SNPs of the *IL-13* gene correlate with long term out-come of INS, we did not see any association between the analysed SNP and the number of relapses, response to medication, or any other feature. *MIF* is counter-regulated by GCs. Vivarelli *et al.* (240) found that the frequency of C allele of SNP rs755622 was higher in Italian patients than in controls and higher

in SRNS than in SSNS. Similarly, Berdeli *et al.* (239) found that GC genotype and C allele was higher in patients than in controls and CC genotype was more frequent in patients with SRNS than in those with SSNS. On the other hand, this association did not rise in a study of Korean patients by Choi *et al.* (249). Similarly, our study didn't reveal any association between rs755622 SNP and INS or any of the clinical parameters. Different studies naturally use patients from different populations and population differences in allele frequencies cannot be ruled out.

Angptl4 is a secretory protein involved in lipid metabolism and its increased expression has been observed in podocytes and circulation in human and experimental INS (229, 295). The genetic variant SNP rs1044250 in exon 6 leads to amino acid change p.T266M and the homozygous C genotype of this variant has been associated with lower plasma Angptl4 levels (296). Recently, Clement *et al.* (297) discovered that increased circulating Angptl4 abates proteinuria but at the cost of inducing hypertriglyceridemia. In our analysis the C allele was more frequent in patients who received other IS drug medication in addition to GCs (72 vs. 54%, p=0,0228) but we did not see association of SNP genotypes to the occurrence of INS or clinical severity of the disorder.

Recently, Okamoto *et al.* (175) found an association between glypican-5 (*GPC5*) gene variants and acquired NS (focal segmental glomerulosclerosis, proteinuric IgA-nephropathy) using a genome wide association study and replication analysis. They showed that glypican-5 is localized on the cell surface membranes of the podocyte and that *GPC5* SNP rs16946160 risk genotype (AA) was associated with higher expression of the protein. In this study, we observed an association between rs16946160 A allele and early disease onset (16 vs. 5%, p=0.0421) but we did not find an association between the SNP and INS in general. It is, however, notable that none of our patients and only one of the controls carried the AA genotype. Okamoto *et al.* (175) found the A allele frequency of controls to be 0.168 and dbSNP (www.ncbi.nlm.nih.gov/snp) puts it at 0.161. In this study, it was only 0.08. Thus, it is possible that due to the frequency differences between populations the association between the risk genotype and INS is not visible in our patients.

5.2.3.2 Glucocorticoid metabolism related molecules

MDR1 gene codes for a membranous P-gp, multi-drug transporter expressed in the proximal tubular cells. Certain SNPs in *MDR1* gene are found to affect the expression of the gene or activity of the protein it codes. The common SNP rs1045642 in exon 26 has garnered a lot of attention. It is a synonymous substitution and it may not be causal itself but linked with another variant or have an effect on DNA structure or RNA stability (276). Of the other two common SNPs in this study rs2032582 does lead to amino acid change, p.Ala899Ser/Thr. This variant is suggested to increase the drug resistance of the

cell (298, 299). The data on the significance of these SNPs from different studies are contradictory. The distribution of rs2032582 genotypes was found to be significantly different in healthy controls compared to patients in Indian and Egyptian populations (250, 276) while studies with Polish and Korean subjects did not find the association (248, 249).

All three *MDR1* SNPs showed association with treatment choices, T allele and TT genotype being more common in patients who needed other IS drugs compared to those who were only medicated with GCs, which indicates that T and TT are associated with more complicated form of the disease. Surprisingly, only rs1045642 showed significant association between genotype distribution and GC responsiveness (T allele was more frequent in poor responders), although it must be noted that only ten of our patients were not responsive to GCs; this small cohort size may affect these results.

We did not discover any difference between the Finnish patients and controls in rs2032582 genotypes. Wasilewska *et al.* (248), Jafar *et al.* (250) and Youssef *et al.* (276) found an association between rs1045642 and NS (allele T and genotype TT was higher in patients) while Choi *et al.* (249) did not. Again, population differences may play a role. Youssef *et al.* (276) compared rs1045642 allele frequencies in their Egyptian control subjects and found that the frequencies (C 66.4%, T 33.6%) were consistent with frequencies previously reported in African populations but different from frequencies found in Caucasian, Asian and Indian populations (250, 300). In this study, these frequencies were nearly opposite (C 37.2%, T 62.8%) of those determined by Yussef *et al.* (276). This may affect the association between rs1045642 genotypes and NS that was observed in Egyptian population but not in Finnish. On the other hand, in our study population, *MDR1* SNP rs1045642 CC genotype showed association with higher age of onset (20 vs. 0%). Youssef *et al.* (276) reported similar association for SNPS rs2032582 as well as rs1045642. Other studies did not show this association at all (248). More work should be put to revealing the population differences in allele and genotype frequencies and what effects those may have on the function of the protein.

NR3C1 codes for GR that that plays a role in the regulation of many biological functions, including responsiveness to GC, and its functional variability may affect the response to GC medication. In some previous studies, G allele of SNP rs4142347, especially as a part of intron B three SNP haplotype, has been associated with increased GC sensitivity (236, 301) while others could not confirm the association (302). In our analysis, we did not see the SNPs association to medical regimes. However, we did find that patients with more than five relapses carried more frequently heterozygous GC genotype than those with less than five relapses (68 vs. 33%). Simultaneously, the amount of both CC and GG homozygotes was reduced in the over five relapses -group. Curiously, the allele distribution between over five relapses and fewer than five relapses -

groups showed no difference. It is unclear if this is functionally relevant finding or merely an artifact.

An interesting new gene in the context of INS is *GLCCI1*. Tantisira *et al.* (243) first showed that SNPs rs37973 and rs37972, which are in linkage disequilibrium, associated with poor responsiveness to GCs in asthmatic patients. Soon afterwards, Nishibori *et al.* (244) showed that Glcci1-protein is highly expressed in glomerular podocytes and its deficiency leads to proteinuria. Based on these findings, Cheong *et al.* (245) looked to see if these SNPs were playing a role in GC responsiveness in NS but could find no association. Similarly, we found no association between the alleles or genotypes of either SNP and GC responsiveness but the frequency of the rs37973 A allele was higher in patients who received other IS drugs compared to those who received only GC medication (67 vs. 50%). Also, the A allele was more frequent in patients with more than five relapses compared to those with fewer (70 vs. 52%). To our knowledge this association has not been looked into in other studies.

5.2.3.3 Haplotype analysis of MDR1 SNPs

In previous studies, Wasilewska *et al.* (248) reported significant association between SNPs rs1128503, rs2032582 and rs1045642 formed haplotype frequencies with steroid response time. Choi *et al.* (249) and Youssef *et al.* (276) also saw this association, although, interestingly, the major haplotype linked with this property varies between studies. We did not find this association and Cizmarikova *et al.* (277) reached similar conclusion. The most abundant haplotypes of our study were TTT and CGC. These two were predominant in patient samples with combined allele frequency of 70%. The remaining 30% was distributed between eight other haplotypes, none of them reaching 10% frequency. In control patients TTT and CGC were also the most common haplotypes but not as prevalent as the distribution was more diverse, five haplotypes had over 10% frequency. Previous studies have also shown that TTT and CGC are prevalent haplotypes both in patients and in control subjects (248, 249, 276, 277). Interestingly, Choi *et al.* (249) and Youssef *et al.* (276) found haplotype TGC to have a frequency equal to TTT and CGC, and have association to steroid responsiveness while Cizmarikova *et al.* (277) found the frequency of TGC to be under 3% in both patients and in controls and have no association to any clinical attribute. Our results are similar to the latter study as TGC frequency was 7.8% in control samples and 4.2% in patients. Again it must be noted that these differences may be caused by differences in allele frequencies between populations

5.3 Podocyte protein coding genes in Finnish familial SRNS (III)

5.3.1 Rare clinical course of familial SRNS

Familial SRNS with causative *NPHS2* variants is inherited recessively (OMIM 600995). The disease in the Family AM follows dominant inheritance pattern. Nevertheless, all affected family members carried heterozygous *NPHS2* deletion c.988_989delCT. None of the healthy family members did.

Typically, *NPHS2* variants lead to NS with onset before six years of age and development to ESRD in first decade of life (303). The course of the dominant SRNS usually has later onset in adolescence or even in adulthood (304, 305). In Family AM the disease onset is during early childhood but the slow progression and ESRD in adulthood sets it apart from typical *NPHS2* cases. In a way, the disease course in Family AM combines features from both dominant and recessive forms of the SRNS.

5.3.2 NPHS2 in the familial NS

The *NPHS2* sequence was analysed in several ways. First, it was among the genes we analysed using direct sequencing of exons and exon/intron boundaries, and then it was included to the NS gene panel that checked relevant intronic regions in addition to exon and exon/intron boundaries, and WGS analysis that covered all of the intronic and control regions in addition to the coding ones. In all of these analyses c.988_989delCT was the only variant found that was carried only by the affected patients in the family and none of their healthy family members or unrelated controls.

As the NS in the family AM follows dominant inheritance pattern it is expected that the causative variant is a heterozygous one. However, that the variant should be in *NPHS2* is a surprise.

To the best of our knowledge this is the first time a *NPHS2* variant is suggested to cause dominant NS. While numerous studies report patients with a single heterozygous variant the second one is assumed to go undetected (i.e. lying in the introns, promoter or in other control segments) or the found variant is dismissed as inconsequential (303). Also we suspected that the deletion c.988_989delCT would be only one of two variants and thus carried out several types of sequence analyses to find out if the affected patients had a second variant that would contribute to compound heterozygous state. As said, a second variant in the other allele of *NPHS2* was not discovered. One intronic

variant was found in all affected included in the analysis, but that SNP is located in the same allele as the deletion and is inherited together with it. The intronic variant is carried also by I-1, the healthy mother of the first affected patient of the family. The second allele appears to be without significant variants.

Disease course affected by the specific *NPHS2* variants is not a unique phenomenon. Congenital and infantile onset is common with variants p.R138Q, p.R168H, and truncating variants (183, 306). Milder course with delayed onset and prolonged progression is also reported, and often associated with variant p.R229Q. Compound heterozygous cases where p.R229Q is one of the variants, the mean age of diagnosis is 17 years and ESRD is reached in 26 years on average (8). Homozygous p.R229Q seems not to cause SRNS (189, 307). Some suggest that p.R229Q acts a modifier for variants in other genes (189) while others claim, as mentioned before, its effects depend on the specific variant it is coupled with: p.R229Q is pathogenic when the other allele carries a variant in exon 7 or 8 (190). The course of SRNS seem to be affected by the specific variants in *NPHS2*; it is not just a question of a variant being pathogenic or benign.

Wild type podocin has 384 amino acid residues. The c.988_989delCT truncates the C-terminus of the podocin leaving a protein of 343 amino acids and of these the last 14 are incorrectly formed by the frame shift. The C-terminus of podocin is thought to be involved in the protein binding: in the homodimerization of podocin as well as in interactions with other proteins, such as nephrin and CD2AP (308). Often the protein-protein interactions are facilitated by amino acid residue proline and c.988_989delCT truncation erases nine proline residues of the podocin. We carried out an immunoprecipitation analysis, which showed that the binding of truncated podocin to nephrin was not much altered from the wild type. Whether the homodimerization or other interactions are disturbed remains to be studied. Besides the erasure of the proline residues, The PredictProtein service estimates that the truncation causes small changes to the secondary and tertiary structures of the protein. This may lead to a situation where the truncated podocin may have a modest negative impact on the glomerular filtration barrier, which then leads to the exceptionally slow progression of the disease. This is, of course, mere speculation based on this data. More experiments are required to illuminate how the function of podocin is affected by the deletion c.988_989delCT.

5.3.3 Other podocyte genes in familial SRNS

In order to examine the possibility that the deletion c.988_989delCT is not the disease-causing variant in family AM, we carried out sequence analysis of other genes coding for podocyte proteins with emphasis on genes involved in dominant NS. Few variants were discovered but none that co-segregated with

the disease phenotype. This further emphasizes the possible role of c.988_989delCT as a causative variant.

5.4 Podocyte Proteins in kidney disease (IV)

The cause of CNF is the nucleotide variants in *NPHS1* leading to disruption of the amino acid sequence and absence of nephrin from the SD resulting in the failure of the structure (85). Using immunohistochemistry and CNF tissue sections, we looked into the changes in the expression of various podocyte-expressed proteins to see how the lack of this essential structural and signaling protein affects other cellular aspects of the podocyte. We also examined the expression of few proteins in the podocytes of patients with MCNS to see if same cellular events happen in both diseases.

5.4.1 Slit diaphragm proteins in CNF

The expression of the SD proteins Neph1 and Fat1 was greatly reduced. This finding is in line with electron microscopic imaging of podocyte foot processes in CNF patients, which show the whole SD structure to be missing. Nephrin is the major component of the structure that forms homodimers to bridges over the slit gap and to which other components of the SD bind to. Neph1 is homologous to nephrin. In addition to its structural role in the SD Neph1 is involved in actin remodeling in injury (92). Mice lacking Neph1 are proteinuric and die perinatally (93) and similarly Fat1 knockout mice undergo foot process effacement, massive proteinuria and perinatal lethality (96, 308). This demonstrated their salient roles in SD function even though in neither of them human pathogenic variants have been found.

Due to the essential function of nephrin as a SD scaffold or back bone on which the other components attach themselves, it makes sense that in its absence the other SD proteins would find it difficult to find their correct places or to anchor themselves properly. The poor expression of these three major SD proteins may explain the massive protein loss in CNF.

5.4.2 Adaptor proteins in CNF

The cytosolic adaptor proteins podocin, NCK1/2 and CD2AP connect the SD to the actin cytoskeleton. Interestingly, their expression was very close to controls in the case of podocin and moderately increased in the case of NCK1/2 and CD2AP. Decreased expression of CD2AP is leads to podocyte injury, apoptosis,

and proteinuria (309, 310). Its moderate up-regulation in CNF glomeruli may reflect the podocyte´s attempt to restore critical cellular functions.

CD2AP and Nck1/2 facilitate actin cytoskeleton maintenance and reorganization that is promoted by their binding to phosphorylated nephrin. When rapid actin polymerization and reorganization is required during development and injury repair, Nck and its actin-binding associates are recruited (110). During a steady state, nephrin/CD2AP binding predominates (111, 112). It is curious, then, that in the absence of nephrin both Nck1/2 and CD2AP are upregulated modestly but CD2AP upregulation is over twice of that of Nck1/2 (3.3- vs. 1.4-fold). Whether their expression is independent of nephrin or if the modest increase in their staining is due to a reaction to SD failure in attempt to maintain the cytoskeletal architecture requires further research.

All in all, adaptor proteins play a key role in correct localization of the SD proteins but the opposite does not appear to be true: the absence of nephrin and consequent reduction in other SD components does not greatly affect the adaptor protein staining. However, the precision of immunohistochemistry and light microscopy is not high enough to pinpoint the exact localization of the adapter proteins in the foot processes; there may be alterations that this experiment did not reveal.

5.4.3 Actin cytoskeleton regulating proteins in CNF

Of the three actin cytoskeleton regulating proteins ACTN4 and INF2 showed mildly increased staining while N-WASp staining was not significantly altered. Variants in ACTN4 coding gene are found in patients with AD form of SRNS and previously the upregulation of the protein has been seen in membranous nephropathy (311). Variants in INF2 are also associated with dominantly inherited SRNS and its mouse knockout models lead to actin reorganization. It also plays a role in lipid raft related trafficking of SD proteins (203). It is possible that the upregulation of INF2 is a reaction to the absence of nephrin; a desperate and doomed effort to counteract the failure to form the SD. However, the increases in the staining are not large and how they affect the actin cytoskeleton is not clear.

5.4.4 Basel and apical domain proteins in CNF

Podocytes are attached to the GBM mainly by the α3β1-integrin complex in the basal membrane domain of the foot processes (312). Mutations in *ITGA3,* coding for integrin-α3, lead to CNS with skin and lung disease (158) In our study, α3-integrin staining showed a small increase in CNF glomeruli. However,

the significance of this slight alteration in staining is still unclear. αβ-dystroglycan complex also binds components of the GBM and is thought to play a role in the attachment of the foot processes to the GBM (56). Its expression pattern is altered in experimental proteinuric models and dystroglycan-null mice show moderate foot process effacement (61, 313). However, the alterations are so slight that some investigators suggest its function in the podocyte is mainly superfluous (62). Our results show that in CNF β-dystroglycan staining was moderately reduced. However, as we analysed the staining using light microscopy, it is difficult to say if the localization of the protein is exactly as it is in WT protein.

One of the end-results of podocyte damage is thought to be podocyte detachment from the GBM and it has been suggested that the foot process effacement is a protective mechanism to save the podocytes from detachment. While the effacement of podocyte foot processes occurs in CNF glomerulus, the detachment from the GBM seems to be a rare event (145, 315). It is possible that the increased expression of α3β1-complex in the flattened foot processes adds to their “stickiness” to the GBM, and thus fights against the detachment. In this study, the nearly unaffected staining of podocalyxin, which in immunohistochemistry is often used as a podocyte marker, speaks against significant podocyte loss.

5.4.5 Podocyte proteins in MCNS

While much is known about the molecular basis of CNF and other inherited nephrotic syndromes, the role of podocytes in MCNS is still unclear. Podocyte effacement is seen in EM, but its correlation to proteinuric episodes is not accurate and it is still not clear whether these changes seen in EM are the cause or consequence of proteinuria (217). In immunohistochemistry, nephrin and podocin expression in MCNS kidneys has shown some variation from quite normal to significantly reduced (170, 217, 315). Mao et al. (315) studied the expression profiles of nephrin, podocin and CD2AP at mRNA level in Chinese children with MCNS and found podocin expression not to be affected and CD2AP to be lower in MCNS. We found CD2AP to be downregulated as well, but the difference was insignificantly slight. In fact in our study, all the perceived changes in expression were small, which in case of Neph1 is in sharp contrast to the findings in CNF. No statistically significant changes were found between MCNS with and without proteinuria groups. These findings suggest that the molecular architecture of the podocyte foot process is quite well preserved in MCNS. However, this does not rule out functional defect in the barrier.

6. Conclusion

This thesis concentrates on the analysis of genetic and clinical features to discover causative variants and predictive markers in childhood NS. We also looked into the effects of a known genetic injury to the podocyte structure. The main conclusions of this thesis study are:

1. The outcome in MCNS is quite good. Genetics play a smaller role in its background than in some other forms of NS and reliably predicting its course at the time of onset is currently not possible.

2. The genes coding for proteins involved in glucocorticoid metabolism, especially *MDR1*, probably play a role in modifying the INS phenotype but defining that role is challenging. Differences in allele frequencies between populations may hold significance.

3. The specific *NPHS2* variants have surprisingly large effect on the disease phenotype, affecting the age of onset, disease progression and even the mode of inheritance. The nature of the variant must be considered when carrying out a genetic analysis.

4. Podocyte structure is maintained by communication and interaction between copious amount of proteins, and effects of the removal of such an involved protein as nephrin spreads to all compartments of the cell, but is especially damaging to the other SD proteins. However, considering how pronounced the observed damage to podocyte foot process structure in absence of nephrin is the magnitude of change in podocyte protein expression appears to be curiously moderate.

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References

1. Nyengaard JR, Bendtsen TF. Glomerular number and size in relation to age, kidney weight, and body surface in normal man. Anat Rec. 1992 Feb;232(2):194-201
2. Renkin EM, Robinson RR. Glomerular filtration. N Engl J Med. 1974 Apr 4;290(14):785-92. Review
3. Somlo S, Mundel P. Getting a foothold in nephrotic syndrome. Nat Genet. 2000 Apr;24(4):333-5
4. Pavenstädt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. Physiol Rev. 2003 Jan;83(1):253-307. Review
5. Harvey SJ, Miner JH. Revisiting the glomerular charge barrier in the molecular era. Curr Opin Nephrol Hypertens. 2008 Jul;17(4):393-8
6. Haraldsson B, Nyström J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. Physiol Rev. 2008 Apr;88(2):451-87
7. Hallmann R, Horn N, Selg M, Wendler O, Pausch F, Sorokin LM. Expression and function of laminins in the embryonic and mature vasculature. Physiol Rev. 2005 Jul;85(3):979-1000. Review
8. Machuca E, Hummel A, Nevo F, Dantal J, Martinez F, Al-Sabban E, Baudouin V, Abel L, Grünfeld JP, Antignac C. Clinical and epidemiological assessment of steroid-resistant nephrotic syndrome associated with the NPHS2 R229Q variant. Kidney Int. 2009 Apr;75(7):727-35
9. Miner JH. The glomerular basement membrane. Exp Cell Res. 2012 May 15;318(9):973-8
10. Satchell SC, Braet F. Glomerular endothelial cell fenestrations: an integral component of the glomerular filtration barrier. Am J Physiol Renal Physiol. 2009 May;296(5):F947-56
11. Ichimura K, Stan RV, Kurihara H, Sakai T. Glomerular endothelial cells form diaphragms during development and pathologic conditions. J Am Soc Nephrol. 2008 Aug;19(8):1463-71
12. Weinbaum S, Tarbell JM, Damiano ER. The structure and function of the endothelial glycocalyx layer. Annu Rev Biomed Eng. 2007;9:121-67. Review
13. Pries AR, Secomb TW, Gaehtgens P. The endothelial surface layer. Pflugers Arch. 2000 Sep;440(5):653-66. Review
14. Satchell S. The role of the glomerular endothelium in albumin handling. Nat Rev Nephrol. 2013 Dec;9(12):717-25
15. Dane MJ, van den Berg BM, Avramut MC, Faas FG, van der Vlag J, Rops AL, Ravelli RB, Koster BJ, van Zonneveld AJ, Vink H, Rabelink TJ. Glomerular endothelial surface layer acts as a barrier against albumin filtration. Am J Pathol. 2013 May;182(5):1532-40

16. Levick JR, Smaje LH. An analysis of the permeability of a fenestra. Microvasc Res. 1987 Mar;33(2):233-56
17. Salmon AH, Satchell SC. Endothelial glycocalyx dysfunction in disease: albuminuria and increased microvascular permeability. J Pathol. 2012 Mar;226(4):562-74
18. Satchell SC, Anderson KL, Mathieson PW. Angiopoietin 1 and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties. J Am Soc Nephrol. 2004 Mar;15(3):566-74
19. Salmon AH, Neal CR, Sage LM, Glass CA, Harper SJ, Bates DO. Angiopoietin-1 alters microvascular permeability coefficients in vivo via modification of endothelial glycocalyx. Cardiovasc Res. 2009 Jul 1;83(1):24-33
20. Foster RR, Slater SC, Seckley J, Kerjaschki D, Bates DO, Mathieson PW, Satchell SC. Vascular endothelial growth factor-C, a potential paracrine regulator of glomerular permeability, increases glomerular endothelial cell monolayer integrity and intracellular calcium. Am J Pathol. 2008 Oct;173(4):938-48
21. Sison K, Eremina V, Baelde H, Min W, Hirashima M, Fantus IG, Quaggin SE. Glomerular structure and function require paracrine, not autocrine, VEGF-VEGFR-2 signaling. J Am Soc Nephrol. 2010 Oct;21(10):1691-701
22. Advani A. Vascular endothelial growth factor and the kidney: something of the marvellous. Curr Opin Nephrol Hypertens. 2014 Jan;23(1):87-92
23. Tarbell JM, Ebong EE. The endothelial glycocalyx: a mechano-sensor and -transducer. Sci Signal. 2008 Oct 7;1(40):pt8.
24. Slater SC, Ramnath RD, Uttridge K, Saleem MA, Cahill PA, Mathieson PW, Welsh GI, Satchell SC. Chronic exposure to laminar shear stress induces Kruppel-like factor 2 in glomerular endothelial cells and modulates interactions with co-cultured podocytes. Int J Biochem Cell Biol. 2012 Sep;44(9):1482-90
25. Timpl R. Structure and biological activity of basement membrane proteins. Eur J Biochem. 1989 Apr 1;180(3):487-502. Review
26. Suh JH, Miner JH. The glomerular basement membrane as a barrier to albumin. Nat Rev Nephrol. 2013 Aug;9(8):470-7. Review
27. Miner JH, Yurchenco PD. Laminin functions in tissue morphogenesis. Annu RevCell Dev Biol. 2004; 20:255-84. Review
28. Colognato H, Yurchenco PD. Form and function: the laminin family of heterotrimers. Dev Dyn. 2000 Jun;218(2):213-34. Review
29. Pöschl E, Schlötzer-Schrehardt U, Brachvogel B, Saito K, Ninomiya Y, Mayer U. Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. Development. 2004 Apr;131(7):1619-28
30. Abrahamson DR, Hudson BG, Stroganova L, Borza DB, St John PL. Cellular origins of type IV collagen networks in developing glomeruli.

J Am Soc Nephrol. 2009 Jul;20(7):1471-9

31. Murshed M, Smyth N, Miosge N, Karolat J, Krieg T, Paulsson M, Nischt R. The absence of nidogen 1 does not affect murine basement membrane formation. Mol Cell Biol. 2000 Sep; 20(18):7007-12
32. Schymeinsky J, Nedbal S, Miosge N, Pöschl E, Rao C, Beier DR, Skarnes WC, Timpl R, Bader BL. Gene structure and functional analysis of the mouse nidogen-2 gene: nidogen-2 is not essential for basement membrane formation in mice. Mol Cell Biol. 2002 Oct; 22(19):6820-30
33. Fox JW, Mayer U, Nischt R, Aumailley M, Reinhardt D, Wiedemann H, Mann K, Timpl R, Krieg T, Engel J, *et al.* Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. EMBO J. 1991 Nov; 10(11):3137-46
34. Bader BL, Smyth N, Nedbal S, Miosge N, Baranowsky A, Mokkapati S, Murshed M, Nischt R. Compound genetic ablation of nidogen 1 and 2 causes basement membrane defects and perinatal lethality in mice. Mol Cell Biol. 2005 Aug; 25(15):6846-56
35. Groffen AJ, Ruegg MA, Dijkman H, van de Velden TJ, Buskens CA, van den Born J, Assmann KJ, Monnens LA, Veerkamp JH, van den Heuvel LP. Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. J Histochem Cytochem. 1998 Jan; 46(1):19-27
36. Kanwar YS, Danesh FR, Chugh SS. Contribution of proteoglycans towards the integrated functions of renal glomerular capillaries: a historical perspective. Am J Pathol. 2007 Jul;171(1):9-13. Review
37. van den Hoven MJ, Wijnhoven TJ, Li JP, Zcharia E, Dijkman HB, Wismans RG, Rops AL, Lensen JF, van den Heuvel LP, van Kuppevelt TH, Vlodavsky I, Berden JH, van der Vlag J. Reduction of anionic sites in the glomerular basement membrane by heparanase does not lead to proteinuria. Kidney Int. 2008 Feb;73(3):278-87
38. Goldberg S, Harvey SJ, Cunningham J, Tryggvason K, Miner JH. Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane. Nephrol Dial Transplant. 2009 Jul;24(7):2044-51
39. Axelsson J, Sverrisson K, Rippe A, Fissell W, Rippe B. Reduced diffusion of charge-modified, conformationally intact anionic Ficoll relative to neutral Ficoll across the rat glomerular filtration barrier in vivo. Am J Physiol Renal Physiol. 2011 Oct;301(4):F708-12
40. Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. Physiol Rev. 2007 Jan;87(1):165-217
41. Sachs N, Sonnenberg A. Cell-matrix adhesion of podocytes in physiology and disease. Nat Rev Nephrol. 2013 Apr;9(4):200-10
42. Kim C, Ye F, Ginsberg MH. Regulation of integrin activation. Annu Rev Cell Dev Biol. 2011;27:321-45
43. Margadant C, Monsuur HN, Norman JC, Sonnenberg A. Mechanisms of

integrin activation and trafficking. Curr Opin Cell Biol. 2011 Oct;23(5):607-14
44. Woods A, Couchman JR. Integrin modulation by lateral association. J Biol Chem. 2000 Aug 11;275(32):24233-6. Review
45. Sachs N, Kreft M, van den Bergh Weerman MA, Beynon AJ, Peters TA, Weening JJ, Sonnenberg A. Kidney failure in mice lacking the tetraspanin CD151. J Cell Biol. 2006 Oct 9;175(1):33-9
46. Fässler R, Meyer M. Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev. 1995 Aug 1;9(15):1896-908
47. Kanasaki K, Kanda Y, Palmsten K, Tanjore H, Lee SB, Lebleu VS, Gattone VH Jr, Kalluri R. Integrin beta1-mediated matrix assembly and signaling are critical for the normal development and function of the kidney glomerulus. Dev Biol. 2008 Jan 15;313(2):584-93
48. Pozzi A, Jarad G, Moeckel GW, Coffa S, Zhang X, Gewin L, Eremina V, Hudson BG, Borza DB, Harris RC, Holzman LB, Phillips CL, Fassler R, Quaggin SE, Miner JH, Zent R. Beta1 integrin expression by podocytes is required to maintain glomerular structural integrity. Dev Biol. 2008 Apr 15;316(2):288-301
49. Girgert R, Martin M, Kruegel J, Miosge N, Temme J, Eckes B, Müller GA, Gross O. Integrin α2-deficient mice provide insights into specific functions of collagen receptors in the kidney. Fibrogenesis Tissue Repair. 2010 Sep 22;3:19
50. Bader BL, Rayburn H, Crowley D, Hynes RO. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. Cell. 1998 Nov 13;95(4):507-19
51. Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Culleré M, Ross FP, Coller BS, Teitelbaum S, Hynes RO. Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest. 1999 Jan;103(2):229-38
52. Mayer G, Boileau G, Bendayan M. Furin interacts with proMT1-MMP and integrin alphaV at specialized domains of renal cell plasma membrane. J Cell Sci. 2003 May 1;116(Pt 9):1763-73
53. Wei C, Möller CC, Altintas MM, Li J, Schwarz K, Zacchigna S, Xie L, Henger A, Schmid H, Rastaldi MP, Cowan P, Kretzler M, Parrilla R, Bendayan M, Gupta V, Nikolic B, Kalluri R, Carmeliet P, Mundel P, Reiser J. Modification of kidney barrier function by the urokinase receptor. Nat Med. 2008 Jan;14(1):55-63.
54. Wei C, El Hindi S, Li J, Fornoni A, Goes N, Sageshima J, Maiguel D, Karumanchi SA, Yap HK, Saleem M, Zhang Q, Nikolic B, Chaudhuri A, Daftarian P, Salido E, Torres A, Salifu M, Sarwal MM, Schaefer F, Morath C, Schwenger V, Zeier M, Gupta V, Roth D, Rastaldi MP, Burke G, Ruiz P, Reiser J. Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. Nat Med. 2011 Jul 31;17(8):952-60.

55. Hayashida T, Jones JC, Lee CK, Schnaper HW. Loss of beta1-integrin enhances TGF-beta1-induced collagen expression in epithelial cells via increased alphavbeta3-integrin and Rac1 activity. J Biol Chem. 2010 Oct 1;285(40):30741-51
56. Raats CJ, van den Born J, Bakker MA, Oppers-Walgreen B, Pisa BJ, Dijkman HB, Assmann KJ, Berden JH. Expression of agrin, dystroglycan, and utrophin in normal renal tissue and in experimental glomerulopathies. Am J Pathol. 2000 May;156(5):1749-65
57. Regele HM, Fillipovic E, Langer B, Poczewki H, Kraxberger I, Bittner RE, Kerjaschki D. Glomerular expression of dystroglycans is reduced in minimal change nephrosis but not in focal segmental glomerulosclerosis. J Am Soc Nephrol. 2000 Mar;11(3):403-12
58. Timpl R, Tisi D, Talts JF, Andac Z, Sasaki T, Hohenester E. Structure and function of laminin LG modules. Matrix Biol. 2000 Aug;19(4):309-17. Review
59. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. J Clin Invest. 2001 Dec;108(11):1583-7
60. Williamson RA, Henry MD, Daniels KJ, Hrstka RF, Lee JC, Sunada Y, Ibraghimov-Beskrovnaya O, Campbell KP. Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. Hum Mol Genet. 1997 Jun;6(6):831-41
61. Kojima K, Nosaka H, Kishimoto Y, Nishiyama Y, Fukuda S, Shimada M, Kodaka K, Saito F, Matsumura K, Shimizu T, Toda T, Takeda S, Kawachi H, Uchida S. Defective glycosylation of α-dystroglycan contributes to podocyte flattening. Kidney Int. 2011 Feb;79(3):311-6
62. Jarad G, Pippin JW, Shankland SJ, Kreidberg JA, Miner JH. Dystroglycan does not contribute significantly to kidney development or function, in health or after injury. Am J Physiol Renal Physiol. 2011 Mar;300(3)
63. Morton MJ, Hutchinson K, Mathieson PW, Witherden IR, Saleem MA, Hunter M. Human podocytes possess a stretch-sensitive, Ca2+-activated K+ channel: potential implications for the control of glomerular filtration. J Am Soc Nephrol. 2004 Dec;15(12):2981-7
64. Ziembicki J, Tandon R, Schelling JR, Sedor JR, Miller RT, Huang C. Mechanical force-activated phospholipase D is mediated by Galpha12/13-Rho and calmodulin-dependent kinase in renal epithelial cells. Am J Physiol Renal Physiol. 2005 Oct;289(4):F826-34
65. Endlich N, Endlich K. Stretch, tension and adhesion - adaptive mechanisms of the actin cytoskeleton in podocytes. Eur J Cell Biol. 2006 Apr;85(3-4):229-34
66. Saleem MA, Zavadil J, Bailly M, McGee K, Witherden IR, Pavenstadt H, Hsu H, Sanday J, Satchell SC, Lennon R, Ni L, Bottinger EP, Mundel

P, Mathieson PW. The molecular and functional phenotype of glomerular podocytes reveals key features of contractile smooth muscle cells. Am J Physiol Renal Physiol. 2008 Oct;295(4):F959-70
67. Welsh GI, Saleem MA. The podocyte cytoskeleton--key to a functioning glomerulus in health and disease. Nat Rev Nephrol. 2011 Oct 25;8(1):14-21
68. Kobayashi N, Reiser J, Kriz W, Kuriyama R, Mundel P. Nonuniform microtubular polarity established by CHO1/MKLP1 motor protein is necessary for process formation of podocytes. J Cell Biol. 1998 Dec 28;143(7):1961-70
69. Kobayashi N, Reiser J, Schwarz K, Sakai T, Kriz W, Mundel P. Process formation of podocytes: morphogenetic activity of microtubules and regulation by protein serine/threonine phosphatase PP2A. Histochem Cell Biol. 2001 Mar;115(3):255-66
70. Faul C, Asanuma K, Yanagida-Asanuma E, Kim K, Mundel P. Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. Trends Cell Biol. 2007 Sep;17(9):428-37
71. Kriz W, Elger M, Mundel P, Lemley KV. Structure-stabilizing forces in the glomerular tuft. J Am Soc Nephrol. 1995 Apr;5(10):1731-9. Review
72. Dominguez R, Holmes KC. Actin structure and function. Annu Rev Biophys. 2011;40:169-86
73. Pelletier O, Pokidysheva E, Hirst LS, Bouxsein N, Li Y, Safinya CR. Structure of actin cross-linked with alpha-actinin: a network of bundles. Phys Rev Lett. 2003 Oct 3;91(14):148102
74. Weins A, Schlondorff JS, Nakamura F, Denker BM, Hartwig JH, Stossel TP, Pollak MR. Disease-associated mutant alpha-actinin-4 reveals a mechanism for regulating its F-actin-binding affinity. Proc Natl Acad Sci U S A. 2007 Oct 9;104(41):16080-5
75. Madrid R, Aranda JF, Rodríguez-Fraticelli AE, Ventimiglia L, Andrés-Delgado L, Shehata M, Fanayan S, Shahheydari H, Gómez S, Jiménez A, Martín-Belmonte F, Byrne JA, Alonso MA. The formin INF2 regulates basolateral-to-apical transcytosis andlumen formation in association with Cdc42 and MAL2. Dev Cell. 2010 May 18;18(5):814-27
76. Brown EJ, Schlöndorff JS, Becker DJ, Tsukaguchi H, Tonna SJ, Uscinski AL, Higgs HN, Henderson JM, Pollak MR. Mutations in the formin gene INF2 cause focal segmental glomerulosclerosis. Nat Genet. 2010 Jan;42(1):72-6
77. Barua M, Brown EJ, Charoonratana VT, Genovese G, Sun H, Pollak MR. Mutations in the INF2 gene account for a significant proportion of familial but not sporadic focal and segmental glomerulosclerosis. Kidney Int. 2013 Feb;83(2):316-22
78. Huber TB, Kwoh C, Wu H, Asanuma K, Gödel M, Hartleben B, Blumer KJ, Miner JH, Mundel P, Shaw AS. Bigenic mouse models of focal

segmental glomerulosclerosis involving pairwise interaction of CD2AP, Fyn, and synaptopodin. J Clin Invest. 2006 May;116(5):1337-45
79. Dai S, Wang Z, Pan X, Wang W, Chen X, Ren H, Hao C, Han B, Chen N. Functional analysis of promoter mutations in the ACTN4 and SYNPO genes in focal segmental glomerulosclerosis. Nephrol Dial Transplant. 2010 Mar;25(3):824-35
80. Huber TB, Benzing T. The slit diaphragm: a signaling platform to regulate podocyte function. Curr Opin Nephrol Hypertens. 2005 May;14(3):211-6. Review
81. Simons K, Toombe D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 2000 Oct;1(1):31-9. Review
82. Cherukuri A, Dykstra M, Pierce SK. Floating the raft hypothesis: lipid rafts play a role in immune cell activation. Immunity. 2001 Jun;14(6):657-60. Review
83. Merscher S, Fornoni A. Podocyte pathology and nephropathy - sphingolipids in glomerular diseases. Front Endocrinol (Lausanne). 2014 Jul 30;5:127
84. Patrakka J, Tryggvason K. Nephrin--a unique structural and signaling protein of the kidney filter. Trends Mol Med. 2007 Sep;13(9):396-403
85. Patrakka J, Kestilä M, Wartiovaara J, Ruotsalainen V, Tissari P, Lenkkeri U, Männikkö M, Visapää I, Holmberg C, Rapola J, Tryggvason K, Jalanko H. Congenital nephrotic syndrome (NPHS1): features resulting from different mutations in Finnish patients. Kidney Int. 2000 Sep;58(3):972-80
86. Verma R, Wharram B, Kovari I, Kunkel R, Nihalani D, Wary KK, Wiggins RC, Killen P, Holzman LB. Fyn binds to and phosphorylates the kidney slit diaphragm component nephrin. J Biol Chem. 2003 Jun 6;278(23):20716-23
87. Kestilä M, Lenkkeri U, Männikkö M, Lamerdin J, McCready P, Putaala H, Ruotsalainen V, Morita T, Nissinen M, Herva R, Kashtan CE, Peltonen L, Holmberg C, Olsen A, Tryggvason K. Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. Mol Cell. 1998 Mar;1(4):575-82
88. Sellin L, Huber TB, Gerke P, Quack I, Pavenstädt H, Walz G. NEPH1 defines a novel family of podocin interacting proteins. FASEB J. 2003 Jan; 17(1):1157
89. Gerke P, Huber TB, Sellin L, Benzing T, Walz G. Homodimerization and heterodimerization of the glomerular podocyte proteins nephrin and NEPH1. J Am Soc Nephrol. 2003 Apr;14(4):918-26
90. Barletta GM, Kovari IA, Verma RK, Kerjaschki D, Holzman LB. Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form cishetero-oligomers. J Biol Chem. 2003 May 23;278(21):19266-71

91. Liu G, Kaw B, Kurfis J, Rahmanuddin S, Kanwar YS, Chugh SS. Neph1 and nephrin interaction in the slit diaphragm is an important determinant of glomerular permeability. J Clin Invest. 2003 Jul;112(2):209-21
92. Garg P, Verma R, Nihalani D, Johnstone DB, Holzman LB. Neph1 cooperates with nephrin to transduce a signal that induces actin polymerization. Mol Cell Biol. 2007 Dec;27(24):8698-712
93. Donoviel DB, Freed DD, Vogel H, Potter DG, Hawkins E, Barrish JP, Mathur BN, Turner CA, Geske R, Montgomery CA, Starbuck M, Brandt M, Gupta A, Ramirez-Solis R, Zambrowicz BP, Powell DR. Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. Mol Cell Biol. 2001 Jul;21(14):4829-36
94. Völker LA, Petry M, Abdelsabour-Khalaf M, Schweizer H, Yusuf F, Busch T, Schermer B, Benzing T, Brand-Saberi B, Kretz O, Höhne M, Kispert A. Comparative analysis of Neph gene expression in mouse and chicken development. Histochem Cell Biol. 2012 Mar;137(3):355-66
95. Moeller MJ, Soofi A, Braun GS, Li X, Watzl C, Kriz W, Holzman LB. Protocadherin FAT1 binds Ena/VASP proteins and is necessary for actin dynamics and cell polarization. EMBO J. 2004 Oct 1;23(19):3769-79
96. Ciani L, Patel A, Allen ND, ffrench-Constant C. Mice lacking the giant protocadherin mFAT1 exhibit renal slit junction abnormalities and a partially penetrant cyclopia and anophthalmia phenotype. Mol Cell Biol. 2003 May;23(10):3575-82
97. Radice GL, Ferreira-Cornwell MC, Robinson SD, Rayburn H, Chodosh LA, Takeichi M, Hynes RO. Precocious mammary gland development in P-cadherin-deficient mice. J Cell Biol. 1997 Nov 17;139(4):1025-32
98. Reiser J, Kriz W, Kretzler M, Mundel P. The glomerular slit diaphragm is a modified adherens junction. J Am Soc Nephrol. 2000 Jan;11(1):1-8
99. Mundel P, Shankland SJ. Podocyte biology and response to injury. J Am Soc Nephrol. 2002 Dec;13(12):3005-15. Review
100. Asanuma K, Mundel P. The role of podocytes in glomerular pathobiology. Clin Exp Nephrol. 2003 Dec;7(4):255-9. Review
101. Roselli S, Gribouval O, Boute N, Sich M, Benessy F, Attié T, Gubler MC, Antignac C. Podocin localizes in the kidney to the slit diaphragm area. Am J Pathol. 2002 Jan;160(1):131-9
102. Huber TB, Simons M, Hartleben B, Sernetz L, Schmidts M, Gundlach E, Saleem MA, Walz G, Benzing T. Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. Hum Mol Genet. 2003 Dec 15;12(24):3397-405
103. Huber TB, Schermer B, Müller RU, Höhne M, Bartram M, Calixto A,

Hagmann H, Reinhardt C, Koos F, Kunzelmann K, Shirokova E, Krautwurst D, Harteneck C, Simons M, Pavenstädt H, Kerjaschki D, Thiele C, Walz G, Chalfie M, Benzing T. Podocin and MEC-2 bind cholesterol to regulate the activity of associated ion channels. Proc Natl Acad Sci U S A. 2006 Nov 14;103(46):17079-86

104. Huber TB, Hartleben B, Kim J, Schmidts M, Schermer B, Keil A, Egger L, Lecha RL, Borner C, Pavenstädt H, Shaw AS, Walz G, Benzing T. Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. Mol Cell Biol. 2003 Jul;23(14):4917-28
105. Reeves W, Caulfield JP, Farquhar MG. Differentiation of epithelial foot processes and filtration slits: sequential appearance of occluding junctions, epithelial polyanion, and slit membranes in developing glomeruli. Lab Invest. 1978 Aug;39(2):90-100
106. Grahammer F, Schell C, Huber TB. The podocyte slit diaphragm--from a thin grey line to a complex signalling hub. Nat Rev Nephrol. 2013 Oct;9(10):587-98
107. Macconi D, Ghilardi M, Bonassi ME, Mohamed EI, Abbate M, Colombi F, Remuzzi G, Remuzzi A. Effect of angiotensin-converting enzyme inhibition on glomerular basement membrane permeability and distribution of zonula occludens-1 in MWF rats. J Am Soc Nephrol. 2000;11:477–489
108. Jones N, Blasutig IM, Eremina V, Ruston JM, Bladt F, Li H, Huang H, Larose L, Li SS, Takano T, *et al.* Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. Nature. 2006;440:818–823
109. Jones N, New LA, Fortino MA, Eremina V, Ruston J, Blasutig IM, Aoudjit L, Zou Y, Liu X, Yu GL, *et al.* Nck proteins maintain the adult glomerular filtration barrier. J Am Soc Nephrol. 2009;20:1533–1543
110. Verma R, Kovari I, Soofi A, Nihalani D, Patrie K, Holzman LB. Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. J Clin Invest. 2006 May;116(5):1346-59
111. Benzing T. Signaling at the slit diaphragm. J Am Soc Nephrol. 2004 Jun;15(6):1382-91. Review
112. Putaala H, Sainio K, Sariola H, Tryggvason K. Primary structure of mouse and rat nephrin cDNA and structure and expression of the mouse gene. J Am Soc Nephrol. 2000 Jun;11(6):991-1001
113. Angers S, Moon RT. Proximal events in Wnt signal transduction. Nat Rev Mol Cell Biol. 2009 Jul;10(7):468-77
114. Kato H, Gruenwald A, Suh JH, Miner JH, Barisoni-Thomas L, Taketo MM, Faul C, Millar SE, Holzman LB, Susztak K. Wnt/β-catenin pathway in podocytes integrates cell adhesion, differentiation, and survival. J Biol Chem. 2011 Jul 22;286(29):26003-15
115. Obeidat M, Obeidat M, Ballermann BJ. Glomerular endothelium: a

porous sieve and formidable barrier. Exp Cell Res. 2012 May 15;318(9):964-72
116. Xu C, Chang A, Hack BK, Eadon MT, Alper SL, Cunningham PN. TNF-mediated damage to glomerular endothelium is an important determinant of acute kidney injury in sepsis. Kidney Int. 2014 Jan;85(1):72-81
117. Jeansson M, Haraldsson B. Morphological and functional evidence for animportant role of the endothelial cell glycocalyx in the glomerular barrier. Am J Physiol Renal Physiol. 2006 Jan;290(1):F111-6
118. Singh A, Fridén V, Dasgupta I, Foster RR, Welsh GI, Tooke JE, Haraldsson B, Mathieson PW, Satchell SC. High glucose causes dysfunction of the human glomerular endothelial glycocalyx. Am J Physiol Renal Physiol. 2011 Jan;300(1):F40-8
119. Nolasco LH, Turner NA, Bernardo A, Tao Z, Cleary TG, Dong JF, Moake JL. Hemolytic uremic syndrome-associated Shiga toxins promote endothelial-cell secretion and impair ADAMTS13 cleavage of unusually large von Willebrand factor multimers. Blood. 2005 Dec 15;106(13):4199-209
120. Noris M, Remuzzi G. Atypical hemolytic-uremic syndrome. N Engl J Med. 2009 Oct 22;361(17):1676-87
121. Powe CE, Levine RJ, Karumanchi SA. Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease. Circulation. 2011 Jun 21;123(24):2856-69
122. Kerr H, Richards A. Complement-mediated injury and protection of endothelium: lessons from atypical haemolytic uraemic syndrome. Immunobiology. 2012 Feb;217(2):195-203
123. Zenker M, Aigner T, Wendler O, Tralau T, Müntefering H, Fenski R, Pitz S, Schumacher V, Royer-Pokora B, Wühl E, Cochat P, Bouvier R, Kraus C, Mark K, Madlon H, Dötsch J, Rascher W, Maruniak-Chudek I, Lennert T, Neumann LM, Reis A. Human laminin beta2 deficiency causes congenital nephrosis with mesangial sclerosis and distinct eye abnormalities. Hum Mol Genet. 2004 Nov 1;13(21):2625-32
124. Matejas V, Hinkes B, Alkandari F, Al-Gazali L, Annexstad E, Aytac MB, Barrow M, Bláhová K, Bockenhauer D, Cheong HI, Maruniak-Chudek I, Cochat P, Dötsch J, Gajjar P, Hennekam RC, Janssen F, Kagan M, Kariminejad A, Kemper MJ, Koenig J, Kogan J, Kroes HY, Kuwertz-Bröking E, Lewanda AF, Medeira A, Muscheites J, Niaudet P, Pierson M, Saggar A, Seaver L, Suri M, Tsygin A, Wühl E, Zurowska A, Uebe S, Hildebrandt F, Antignac C, Zenker M. Mutations in the human laminin beta2 (LAMB2) gene and the associated phenotypic spectrum. Hum Mutat. 2010 Sep;31(9):992-1002
125. Jarad G, Cunningham J, Shaw AS, Miner JH. Proteinuria precedes podocyte abnormalities inLamb2-/- mice, implicating the glomerular basement membrane as an albumin barrier. J Clin Invest. 2006

Aug;116(8):2272-9
126. Hasselbacher K, Wiggins RC, Matejas V, Hinkes BG, Mucha B, Hoskins BE, Ozaltin F, Nürnberg G, Becker C, Hangan D, Pohl M, Kuwertz-Bröking E, Griebel M, Schumacher V, Royer-Pokora B, Bakkaloglu A, Nürnberg P, Zenker M, Hildebrandt F. Recessive missense mutations in LAMB2 expand the clinical spectrum of LAMB2-associated disorders. Kidney Int. 2006 Sep;70(6):1008-12
127. Kagan M, Cohen AH, Matejas V, Vlangos C, Zenker M. A milder variant of Pierson syndrome. Pediatr Nephrol. 2008 Feb;23(2):323-7
128. Savige J, Gregory M, Gross O, Kashtan C, Ding J, Flinter F. Expert guidelines for the management of Alport syndrome and thin basement membrane nephropathy. J Am Soc Nephrol. 2013 Feb;24(3):364-75
129. Harvey SJ, Jarad G, Cunningham J, Rops AL, van der Vlag J, Berden JH, Moeller MJ, Holzman LB, Burgess RW, Miner JH. Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity. Am J Pathol. 2007 Jul;171(1):139-52
130. George B, Verma R, Soofi AA, Garg P, Zhang J, Park TJ, Giardino L, Ryzhova L, Johnstone DB, Wong H, Nihalani D, Salant DJ, Hanks SK, Curran T, Rastaldi MP, Holzman LB. Crk1/2-dependent signaling is necessary for podocyte foot process spreading in mouse models of glomerular disease. J Clin Invest. 2012 Feb 1;122(2):674-92
131. Farquhar MG, Vernier RL, Good RA. An electron microscope study of the glomerulus in nephrosis, glomerulonephritis, and lupus erythematosus. J Exp Med. 1957 Nov 1;106(5):649-60
132. Caulfield JP, Reid JJ, Farquhar MG. Alterations of the glomerular epithelium in acute aminonucleoside nephrosis. Evidence for formation of occluding junctions and epithelial cell detachment. Lab Invest. 1976 Jan;34(1):43-59
133. Kriz W, Shirato I, Nagata M, LeHir M, Lemley KV. The podocyte's response to stress: the enigma of foot process effacement. Am J Physiol Renal Physiol. 2013 Feb 15;304(4):F333-47
134. Seiler MW, Rennke HG, Venkatachalam MA, Cotran RS. Pathogenesis of polycation-induced alterations ("fusion") of glomerular epithelium. Lab Invest. 1977 Jan; 36(1):48-61
135. Shirato I, Hosser H, Kimura K, Sakai T, Tomino Y, Kriz W. The development of focal segmental glomerulosclerosis in masugi nephritis is based on progressive podocyte damage. Virchows Arch. 1996 Nov;429(4-5):255-73
136. Shirato I. Podocyte process effacement in vivo. Microsc Res Tech. 2002 May 15;57(4):241-6
137. Smoyer WE, Mundel P, Gupta A, Welsh MJ. Podocyte alpha-actinin induction precedes foot process effacement in experimental nephrotic syndrome. Am J Physiol. 1997 Jul;273(1 Pt 2):F150-7

138. Schordan S, Schordan E, Endlich K, Endlich N. AlphaV-integrins mediate the mechanoprotective action of osteopontin in podocytes. Am J Physiol Renal Physiol. 2011 Jan;300(1):F119-32
139. Berger K, Moeller MJ. Podocytopenia, parietal epithelial cells and glomerulosclerosis. Nephrol Dial Transplant. 2014 May;29(5):948-50
140. Schiffer M, Bitzer M, Roberts IS, Kopp JB, ten Dijke P, Mundel P, Böttinger EP. Apoptosis in podocytes induced by TGF-beta and Smad7. J Clin Invest. 2001 Sep;108(6):807-16
141. Stieger N, Worthmann K, Schiffer M. The role of metabolic and haemodynamic factors in podocyte injury in diabetes. Diabetes Metab Res Rev. 2011 Mar;27(3):207-15
142. Lee HS. Mechanisms and consequences of TGF-ß overexpression by podocytes in progressive podocyte disease. Cell Tissue Res. 2012 Jan;347(1):129-40. Review
143. Tharaux PL, Huber TB. How many ways can a podocyte die? Semin Nephrol. 2012 Jul;32(4):394-404
144. Kuusniemi AM, Merenmies J, Lahdenkari AT, Holmberg C, Salmela K, Karikoski R, Rapola J, Jalanko H. Glomerular sclerosis in kidneys with congenital nephritic syndrome (NPHS1). Kidney Int. 2006 Oct;70(8):1423-31
145. Lahdenkari AT, Lounatmaa K, Patrakka J, Holmberg C, Wartiovaara J, Kestilä M, Koskimies O, Jalanko H. Podocytes are firmly attached to glomerular basement membrane in kidneys with heavy proteinuria. J Am Soc Nephrol. 2004; 15(10):2611-8
146. Eddy AA and Symons JM. Nephrotic syndrome in childhood. Lancet 362: 629-39, 2003
147. Niaudet P. Steroid-sensitive idiopathic nephrotic syndrome. In Avner E. D H.W.E., Niaudet P (Ed.) Pediatric Nephrology. Baltimore: Lippincott, Williams & Wilkins, 2004, pp. 543-556
148. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, Dahan K, Gubler MC, Niaudet P, Antignac C. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat Genet. 2000 Apr;24(4):349-54
149. Hinkes B, Wiggins RC, Gbadegesin R, Vlangos CN, Seelow D, Nürnberg G, Garg P, Verma R, Chaib H, Hoskins BE, Ashraf S, Becker C, Hennies HC, Goyal M, Wharram BL, Schachter AD, Mudumana S, Drummond I, Kerjaschki D, Waldherr R, Dietrich A, Ozaltin F, Bakkaloglu A, Cleper R, Basel-Vanagaite L, Pohl M, Griebel M, Tsygin AN, Soylu A, Müller D, Sorli CS, Bunney TD, Katan M, Liu J, Attanasio M, O'toole JF, Hasselbacher K, Mucha B, Otto EA, Airik R, Kispert A, Kelley GG, Smrcka AV, Gudermann T, Holzman LB, Nürnberg P, Hildebrandt F. Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be

reversible. Nat Genet. 2006 Dec;38(12):1397-405
150. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, Miner JH, Shaw AS. Congenital nephrotic syndrome in mice lacking CD2-associated protein. Science. 1999 Oct 8;286(5438):312-5
151. Winn MP, Conlon PJ, Lynn KL, Farrington MK, Creazzo T, Hawkins AF, Daskalakis N, Kwan SY, Ebersviller S, Burchette JL, Pericak-Vance MA, Howell DN, Vance JM, Rosenberg PB. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. Science. 2005 Jun 17;308(5729):1801-4
152. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, Mathis BJ, Rodríguez-Pérez JC, Allen PG, Beggs AH, Pollak MR. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. Nat Genet. 2000 Mar;24(3):251-6
153. Kopp JB, Smith MW, Nelson GW, Johnson RC, Freedman BI, Bowden DW, Oleksyk T, McKenzie LM, Kajiyama H, Ahuja TS, Berns JS, Briggs W, Cho ME, Dart RA, Kimmel PL, Korbet SM, Michel DM, Mokrzycki MH, Schelling JR, Simon E, Trachtman H, Vlahov D, Winkler CA. MYH9 is a major-effect risk gene for focal segmental glomerulosclerosis. Nat Genet. 2008 Oct;40(10):1175-84
154. Akilesh S, Suleiman H, Yu H, Stander MC, Lavin P, Gbadegesin R, Antignac C, Pollak M, Kopp JB, Winn MP, Shaw AS. Arhgap24 inactivates Rac1 in mouse podocytes, and a mutant form is associated with familial focal segmental glomerulosclerosis. J Clin Invest. 2011 Oct;121(10):4127-37
155. Mele C, Iatropoulos P, Donadelli R, Calabria A, Maranta R, Cassis P, Buelli S, Tomasoni S, Piras R, Krendel M, Bettoni S, Morigi M, Delledonne M, Pecoraro C, Abbate I, Capobianchi MR, Hildebrandt F, Otto E, Schaefer F, Macciardi F, Ozaltin F, Emre S, Ibsirlioglu T, Benigni A, Remuzzi G, Noris M; PodoNet Consortium. MYO1E mutations and childhood familial focal segmental glomerulosclerosis. N Engl J Med. 2011 Jul 28;365(4):295-306
156. Gupta IR, Baldwin C, Auguste D, Ha KC, El Andalousi J, Fahiminiya S, Bitzan M, Bernard C, Akbari MR, Narod SA, Rosenblatt DS, Majewski J, Takano T. ARHGDIA: a novel gene implicated in nephrotic syndrome. J Med Genet. 2013 May;50(5):330-8
157. Gbadegesin RA, Hall G, Adeyemo A, Hanke N, Tossidou I, Burchette J, Wu G, Homstad A, Sparks MA, Gomez J, Jiang R, Alonso A, Lavin P, Conlon P, Korstanje R, Stander MC, Shamsan G, Barua M, Spurney R, Singhal PC, Kopp JB, Haller H, Howell D, Pollak MR, Shaw AS, Schiffer M, Winn MP. Mutations in the gene that encodes the F-actin binding protein anillin cause FSGS. J Am Soc Nephrol. 2014 Sep;25(9):1991-2002
158. Has C, Spartà G, Kiritsi D, Weibel L, Moeller A, Vega-Warner V, Waters A, He Y, Anikster Y, Esser P, Straub BK, Hausser I, Bockenhauer D, Dekel B, Hildebrandt F, Bruckner-Tuderman L, Laube GF. Integrin

α3 mutations with kidney, lung, and skin disease. N Engl J Med. 2012 Apr 19;366(16):1508-14
159. Kambham N, Tanji N, Seigle RL, Markowitz GS, Pulkkinen L, Uitto J, D'Agati VD. Congenital focal segmental glomerulosclerosis associated with beta4 integrin mutation and epidermolysis bullosa. Am J Kidney Dis. 2000 Jul;36(1):190-6
160. Gee HY, Ashraf S, Wan X, Vega-Warner V, Esteve-Rudd J, Lovric S, Fang H, Hurd TW, Sadowski CE, Allen SJ, Otto EA, Korkmaz E, Washburn J, Levy S, Williams DS, Bakkaloglu SA, Zolotnitskaya A, Ozaltin F, Zhou W, Hildebrandt F. Mutations in EMP2 cause childhood-onset nephrotic syndrome. Am J Hum Genet. 2014 Jun 5;94(6):884-90
161. Barbaux S, Niaudet P, Gubler MC, Grünfeld JP, Jaubert F, Kuttenn F, Fékété CN, Souleyreau-Therville N, Thibaud E, Fellous M, McElreavey K. Donor splice-site mutations in WT1 are responsible for Frasier syndrome. Nat Genet. 1997 Dec;17(4):467-70
162. Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, Pepicelli CV, Gan L, Lee B, Johnson RL. Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat Genet. 1998 May;19(1):51-5
163. Dreyer SD, Zhou G, Baldini A, Winterpacht A, Zabel B, Cole W, Johnson RL, Lee B. Mutations in LMX1B cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. Nat Genet. 1998 May;19(1):47-50
164. Boerkoel CF, Takashima H, John J, Yan J, Stankiewicz P, Rosenbarker L, André JL, Bogdanovic R, Burguet A, Cockfield S, Cordeiro I, Fründ S, Illies F, Joseph M, Kaitila I, Lama G, Loirat C, McLeod DR, Milford DV, Petty EM, Rodrigo F, Saraiva JM, Schmidt B, Smith GC, Spranger J, Stein A, Thiele H, Tizard J, Weksberg R, Lupski JR, Stockton DW. Mutant chromatin remodeling protein SMARCAL1 causes Schimke immuno-osseous dysplasia. Nat Genet. 2002 Feb;30(2):215-20
165. Kurogouchi F, Oguchi T, Mawatari E, Yamaura S, Hora K, Takei M, Sekijima Y, Ikeda Si, Kiyosawa K. A case of mitochondrial cytopathy with a typical point mutation for MELAS, presenting with severe focal-segmental glomerulosclerosis as main clinical manifestation. Am J Nephrol. 1998;18(6):551-6
166. Heeringa SF, Chernin G, Chaki M, Zhou W, Sloan AJ, Ji Z, Xie LX, Salviati L, Hurd TW, Vega-Warner V, Killen PD, Raphael Y, Ashraf S, Ovunc B, Schoeb DS, McLaughlin HM, Airik R, Vlangos CN, Gbadegesin R, Hinkes B, Saisawat P, Trevisson E, Doimo M *et al.* COQ6 mutations in human patients produce nephrotic syndrome with sensorineural deafness. J Clin Invest. 2011 May;121(5):2013-24
167. Diomedi-Camassei F, Di Giandomenico S, Santorelli FM, Caridi G, Piemonte F, Montini G, Ghiggeri GM, Murer L, Barisoni L, Pastore A,

Muda AO, Valente ML, Bertini E, Emma F. COQ2 nephropathy: a newly described inherited mitochondriopathy with primary renal involvement. J Am Soc Nephrol. 2007 Oct;18(10):2773-80

168. López LC, Schuelke M, Quinzii CM, Kanki T, Rodenburg RJ, Naini A, Dimauro S, Hirano M. Leigh syndrome with nephropathy and CoQ10 deficiency due to decaprenyl diphosphate synthase subunit 2 (PDSS2) mutations. Am J Hum Genet. 2006 Dec;79(6):1125-9

169. Berkovic SF, Dibbens LM, Oshlack A, Silver JD, Katerelos M, Vears DF, Lüllmann-Rauch R, Blanz J, Zhang KW, Stankovich J, Kalnins RM, Dowling JP, Andermann E, Andermann F, Faldini E, D'Hooge R, Vadlamudi L, Macdonell RA, Hodgson BL, Bayly MA, Savige J, Mulley JC, Smyth GK, Power DA, Saftig P, Bahlo M. Array-based gene discovery with three unrelated subjects shows SCARB2/LIMP-2 deficiency causes myoclonus epilepsy and glomerulosclerosis. Am J Hum Genet. 2008 Mar;82(3):673-8

170. Agrawal V, Prasad N, Jain M, Pandey R. Reduced podocin expression in minimal change disease and focal segmental glomerulosclerosis is related to the level of proteinuria. Clin Exp Nephrol. 2013 Dec;17(6):811-8

171. Cooperstone BG, Friedman A, Kaplan BS. Galloway-Mowat syndrome of abnormal gyral patterns and glomerulopathy. Am J Med Genet. 1993 Aug 15;47(2):250-4. Review

172. van der Knaap MS, Wevers RA, Monnens L, Jakobs C, Jaeken J, van Wijk JA. Congenital nephrotic syndrome: a novel phenotype of type I carbohydrate-deficient glycoprotein syndrome. J Inherit Metab Dis. 1996;19(6):787-91

173. Kranz C, Denecke J, Lehle L, Sohlbach K, Jeske S, Meinhardt F, Rossi R, Gudowius S, Marquardt T. Congenital disorder of glycosylation type Ik (CDG-Ik): a defect of mannosyltransferase I. Am J Hum Genet. 2004 Mar;74(3):545-51

174. Ozaltin F, Ibsirlioglu T, Taskiran EZ, Baydar DE, Kaymaz F, Buyukcelik M, Kilic BD, Balat A, Iatropoulos P, Asan E, Akarsu NA, Schaefer F, Yilmaz E, Bakkaloglu A; PodoNet Consortium. Disruption of PTPRO causes childhood-onset nephrotic syndrome. Am J Hum Genet. 2011 Jul 15;89(1):139-47

175. Okamoto K, Tokunaga K, Doi K, Fujita T, Suzuki H, Katoh T, Watanabe T, Nishida N, Mabuchi A, Takahashi A, Kubo M, Maeda S, Nakamura Y, Noiri E. Common variation in GPC5 is associated with acquired nephrotic syndrome. Nat Genet. 2011; 43(5):459-63

176. Bolk S, Puffenberger EG, Hudson J, Morton DH, Chakravarti A. Elevated frequency and allelic heterogeneity of congenital nephrotic syndrome, Finnish type, in the old order Mennonites. Am J Hum Genet. 1999 Dec;65(6):1785-90

177. Holmberg C, Tryggvason K, Kestilä M and Jalanko H. Congenital Nephrotic Syndrome. In Avner E H. W., Niaudet P. (Ed.) Pediatric

Nephrology. Baltimore: Lippincott, Williams & Wilkins, 2004, pp. 503-516
178. Jalanko H. Congenital nephrotic syndrome. Pediatr Nephrol. 2009 Nov;24(11):2121-8
179. Santín S, García-Maset R, Ruíz P, Giménez I, Zamora I, Peña A, Madrid A, Camacho JA, Fraga G, Sánchez-Moreno A, Cobo MA, Bernis C, Ortiz A, de Pablos AL, Pintos G, Justa ML, Hidalgo-Barquero E, Fernández-Llama P, Ballarín J, Ars E, Torra R; FSGS Spanish Study Group. Nephrin mutations cause childhood- and adult-onset focal segmental glomerulosclerosis. Kidney Int. 2009 Dec;76(12):1268-76
180. Benoit G, Machuca E, Antignac C. Hereditary nephrotic syndrome: a systematic approach for genetic testing and a review of associated podocyte gene mutations. Pediatr Nephrol. 2010 Sep;25(9):1621-32
181. Benoit G, Machuca E, Heidet L, Antignac C. Hereditary kidney diseases: highlighting the importance of classical Mendelian phenotypes. Ann N Y Acad Sci. 2010 Dec;1214:83-98.
182. Hildebrandt F. Genetic kidney diseases. Lancet. 2010 Apr 10;375(9722):1287-95
183. Hinkes BG, Mucha B, Vlangos CN, Gbadegesin R, Liu J, Hasselbacher K, Hangan D, Ozaltin F, Zenker M, Hildebrandt F; Arbeitsgemeinschaft für Paediatrische Nephrologie Study Group. Nephrotic syndrome in the first year of life: two thirds of cases are caused by mutations in 4 genes (NPHS1, NPHS2, WT1, and LAMB2). Pediatrics. 2007 Apr;119(4):e907-19
184. Lovric S, Fang H, Vega-Warner V, Sadowski CE, Gee HY, Halbritter J, Ashraf S, Saisawat P, Soliman NA, Kari JA, Otto EA, Hildebrandt F; Nephrotic Syndrome Study Group. Rapid detection of monogenic causes of childhood-onset steroid-resistant nephrotic syndrome. Clin J Am Soc Nephrol. 2014 Jun 6;9(6):1109-16
185. Sadowski CE, Lovric S, Ashraf S, Pabst WL, Gee HY, Kohl S, Engelmann S, Vega-Warner V, Fang H, Halbritter J, Somers MJ, Tan W, Shril S, Fessi I, Lifton RP, Bockenhauer D, El-Desoky S, Kari JA, Zenker M, Kemper MJ, Mueller D, Fathy HM, Soliman NA; SRNS Study Group, Hildebrandt F. A single-gene cause in 29.5% of cases of steroid-resistant nephrotic syndrome. J Am Soc Nephrol. 2015 Jun;26(6):1279-89
186. Weber S, Gribouval O, Esquivel EL, Morinière V, Tête MJ, Legendre C, Niaudet P, Antignac C. NPHS2 mutation analysis shows genetic heterogeneity of steroid-resistant nephrotic syndrome and low post-transplant recurrence. Kidney Int. 2004 Aug;66(2):571-9
187. Roselli S, Heidet L, Sich M, Henger A, Kretzler M, Gubler MC, Antignac C. Early glomerular filtration defect and severe renal disease in podocin-deficient mice. Mol Cell Biol. 2004 Jan;24(2):550-60
188. Fan Q, Zhang H, Ding J, Liu S, Miao J, Xing Y, Yu Z, Guan N. R168H and V165X mutant podocin might induce different degrees of

podocyte injury via different molecular mechanisms. Genes Cells. 2009 Sep;14(9):1079-90
189. Kerti A, Csohány R, Wagner L, Jávorszky E, Maka E, Tory K. NPHS2 homozygous p.R229Q variant: potential modifier instead of causal effect in focal segmental glomerulosclerosis. Pediatr Nephrol. 2013 Oct;28(10):2061-4
190. Tory K, Menyhárd DK, Woerner S, Nevo F, Gribouval O, Kerti A, Stráner P, Arrondel C, Huynh Cong E, Tulassay T, Mollet G, Perczel A, Antignac C. Mutation-dependent recessive inheritance of NPHS2-associated steroid-resistant nephrotic syndrome. Nat Genet. 2014 Mar;46(3):299-304
191. Akchurin O, Reidy KJ. Genetic causes of proteinuria and nephrotic syndrome: impact on podocyte pathobiology. Pediatr Nephrol. 2015 Feb;30(2):221-33
192. Gigante M, Pontrelli P, Montemurno E, Roca L, Aucella F, Penza R, Caridi G, Ranieri E, Ghiggeri GM, Gesualdo L. CD2AP mutations are associated with sporadic nephritic syndrome and focal segmental glomerulosclerosis (FSGS). Nephrol Dial Transplant. 2009 Jun;24(6):1858-64
193. Kim JM, Wu H, Green G, Winkler CA, Kopp JB, Miner JH, Unanue ER, Shaw AS. CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. Science. 2003 May 23;300(5623):1298-300
194. Löwik MM, Groenen PJ, Pronk I, Lilien MR, Goldschmeding R, Dijkman HB, Levtchenko EN, Monnens LA, van den Heuvel LP. Focal segmental glomerulosclerosis in a patient homozygous for a CD2AP mutation. Kidney Int. 2007 Nov;72(10):1198-203
195. Weins A, Kenlan P, Herbert S, Le TC, Villegas I, Kaplan BS, Appel GB, Pollak MR. Mutational and Biological Analysis of alpha-actinin-4 in focal segmental glomerulosclerosis. J Am Soc Nephrol. 2005 Dec;16(12):3694-701
196. Michaud JL, Chaisson KM, Parks RJ, Kennedy CR. FSGS-associated alpha-actinin-4 (K256E) impairs cytoskeletal dynamics in podocytes. Kidney Int. 2006 Sep;70(6):1054-61
197. Yao J, Le TC, Kos CH, Henderson JM, Allen PG, Denker BM, Pollak MR. Alpha-actinin-4-mediated FSGS: an inherited kidney disease caused by an aggregated and rapidly degraded cytoskeletal protein. PLoS Biol. 2004 Jun;2(6):e167
198. Michaud JL, Lemieux LI, Dubé M, Vanderhyden BC, Robertson SJ, Kennedy CR. Focal and segmental glomerulosclerosis in mice with podocyte-specific expression of mutant alpha-actinin-4. J Am Soc Nephrol. 2003 May;14(5):1200-11
199. Cybulsky AV, Takano T, Papillon J, Bijian K, Guillemette J, Kennedy CR. Glomerular epithelial cell injury associated with mutant alpha-actinin-4. Am J Physiol Renal Physiol. 2009 Oct;297(4):F987-95

200. Boyer O, Benoit G, Gribouval O, Nevo F, Tête MJ, Dantal J, Gilbert-Dussardier B, Touchard G, Karras A, Presne C, Grunfeld JP, Legendre C, Joly D, Rieu P, Mohsin N, Hannedouche T, Moal V, Gubler MC, Broutin I, Mollet G, Antignac C. Mutations in INF2 are a major cause of autosomal dominant focal segmental glomerulosclerosis. J Am Soc Nephrol. 2011 Feb;22(2):239-45
201. Boyer O, Nevo F, Plaisier E, Funalot B, Gribouval O, Benoit G, Cong EH, Arrondel C, Tête MJ, Montjean R, Richard L, Karras A, Pouteil-Noble C, Balafrej L, Bonnardeaux A, Canaud G, Charasse C, Dantal J, Deschenes G, Deteix P, Dubourg O, Petiot P, Pouthier D, Leguern E, Guiochon-Mantel A, Broutin I, Gubler MC, Saunier S, Ronco P, Vallat JM, Alonso MA, Antignac C, Mollet G. INF2 mutations in Charcot-Marie-Tooth disease with glomerulopathy. N Engl J Med. 2011 Dec 22;365(25):2377-88
202. Sun H, Schlondorff JS, Brown EJ, Higgs HN, Pollak MR. Rho activation of mDia formins is modulated by an interaction with inverted formin 2 (INF2). Proc Natl Acad Sci U S A. 2011 Feb 15;108(7):2933-8
203. Sun H, Schlondorff J, Higgs HN, Pollak MR. Inverted formin 2 regulates actin dynamics by antagonizing Rho/diaphanous-related formin signaling. J Am Soc Nephrol. 2013 May;24(6):917-29
204. Gee HY, Saisawat P, Ashraf S, Hurd TW, Vega-Warner V, Fang H, Beck BB, Gribouval O, Zhou W, Diaz KA, Natarajan S, Wiggins RC, Lovric S, Chernin G, Schoeb DS, Ovunc B, Frishberg Y, Soliman NA, Fathy HM, Goebel H, Hoefele J, Weber LT, Innis JW, Faul C, Han Z, Washburn J, Antignac C, Levy S, Otto EA, Hildebrandt F. ARHGDIA mutations cause nephrotic syndrome via defective RHO GTPase signaling. J Clin Invest. 2013 Aug 1;123(8):3243-53
205. Gigante M, Caridi G, Montemurno E, Soccio M, d'Apolito M, Cerullo G, Aucella F, Schirinzi A, Emma F, Massella L, Messina G, De Palo T, Ranieri E, Ghiggeri GM, Gesualdo L. TRPC6 mutations in children with steroid-resistant nephrotic syndrome and atypical phenotype. Clin J Am Soc Nephrol. 2011 Jul;6(7):1626-34
206. Reiser J, Polu KR, Möller CC, Kenlan P, Altintas MM, Wei C, Faul C, Herbert S, Villegas I, Avila-Casado C, McGee M, Sugimoto H, Brown D, Kalluri R, Mundel P, Smith PL, Clapham DE, Pollak MR. TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. Nat Genet. 2005 Jul;37(7):739-44
207. Krall P, Canales CP, Kairath P, Carmona-Mora P, Molina J, Carpio JD, Ruiz P, Mezzano SA, Li J, Wei C, Reiser J, Young JI, Walz K. Podocyte-specific overexpression of wild type or mutant trpc6 in mice is sufficient to cause glomerular disease. PLoS One. 2010 Sep 20;5(9):e12859
208. Jiang L, Ding J, Tsai H, Li L, Feng Q, Miao J, Fan Q. Over-expressing transient receptor potential cation channel 6 in podocytes induces cytoskeleton rearrangement through increases of intracellular Ca2+

and RhoA activation. Exp Biol Med (Maywood). 2011 Feb;236(2):184-93

209. Wang L, Fields TA, Pazmino K, Dai Q, Burchette JL, Howell DN, Coffman TM, Spurney RF. Activation of Galpha q-coupled signaling pathways in glomerular podocytes promotes renal injury. J Am Soc Nephrol. 2005 Dec;16(12):3611-22
210. Kim EY, Anderson M, Wilson C, Hagmann H, Benzing T, Dryer SE. NOX2 interacts with podocyte TRPC6 channels and contributes to their activation by diacylglycerol: essential role of podocin in formation of this complex. Am J Physiol Cell Physiol. 2013 Nov 1;305(9):C960-71
211. Wing MR, Bourdon DM, Harden TK. PLC-epsilon: a shared effector protein in Ras-, Rho-, and G alpha beta gamma-mediated signaling. Mol Interv. 2003 Aug;3(5):273-80
212. Rigothier C, Auguste P, Welsh GI, Lepreux S, Deminière C, Mathieson PW, Saleem MA, Ripoche J, Combe C. IQGAP1 interacts with components of the slit diaphragm complex in podocytes and is involved in podocyte migration and permeability in vitro. PLoS One. 2012;7(5):e37695
213. Patek CE, Little MH, Fleming S, Miles C, Charlieu JP, Clarke AR, Miyagawa K, Christie S, Doig J, Harrison DJ, Porteous DJ, Brookes AJ, Hooper ML, Hastie ND. A zinc finger truncation of murine WT1 results in the characteristic urogenital abnormalities of Denys-Drash syndrome. Proc Natl Acad Sci U S A. 1999 Mar 16;96(6):2931-6
214. Miner JH, Morello R, Andrews KL, Li C, Antignac C, Shaw AS, Lee B. Transcriptional induction of slit diaphragm genes by Lmx1b is required in podocyte differentiation. J Clin Invest. 2002 Apr;109(8):1065-72
215. Löwik MM, Hol FA, Steenbergen EJ, Wetzels JF, van den Heuvel LP. Mitochondrial tRNALeu(UUR) mutation in a patient with steroid-resistant nephrotic syndrome and focal segmental glomerulosclerosis. Nephrol Dial Transplant. 2005 Feb;20(2):336-41
216. Genovese G, Friedman DJ, Ross MD, Lecordier L, Uzureau P, Freedman BI, Bowden DW, Langefeld CD, Oleksyk TK, Uscinski Knob AL, Bernhardy AJ, Hicks PJ, Nelson GW, Vanhollebeke B, Winkler CA, Kopp JB, Pays E, Pollak MR. Association of trypanolytic ApoL1 variants with kidney disease in African Americans. Science. 2010 Aug 13;329(5993):841-5
217. Patrakka J, Lahdenkari AT, Koskimies O, Holmberg C, Wartiovaara J, Jalanko H. The number of podocyte slit diaphragms is decreased in minimal change nephrotic syndrome. Pediatr Res. 2002; 52(3):349-55
218. Koskimies O, Vilska J, Rapola J, Hallman N. Long-term outcome of primary nephrotic syndrome. Arch Dis Child. 1982 Jul;57(7):544-8
219. Lewis MA, Baildom EM, Davis N, Houston IB and Postlethwaite RJ.

Nephritic syndrome: from toddlers to twenties. Lancet 1989 1: 255-9
220. Cameron JS. The nephrotic syndrome and its complications. Am J Kidney Dis 1987 10:157-71
221. Van den Berg JG and Weening JJ. Role of the immunesystem in the pathogenesis of idiopathic nephrotic syndrome. Clin Sci (Lond) 107: 125-36, 2004
222. Grimbert P, Audard V, Remy P, Lang P, Sahali D. Recent approaches to the pathogenesis of minimal-change nephrotic syndrome. Nephrol Dial Transplant. 2003 Feb;18(2):245-8
223. Yap HK, Yip WC, Lee BW, Ho TF, Teo J, Aw SE, Tay JS. The incidence of atopy in steroid-responsive nephrotic syndrome: clinical and immunological parameters. Ann Allergy. 1983 Dec;51(6):590-4
224. Soeiro EM, Koch VH, Fujimura MD, Okay Y. Influence of nephrotic state on the infectious profile in childhood idiopathic nephrotic syndrome. Rev Hosp Clin Fac Med Sao Paulo. 2004 Oct;59(5):273-8
225. Zhang H, Wang Z, Dong L, Guo Y, Wu J, Zhai S. New insight into the pathogenesis of minimal change nephrotic syndrome: Role of the persistence of respiratory tract virus in immune disorders. Autoimmun Rev. 2016 Feb 11. pii: S1568-9972(16)30033-7. Review
226. Ikeuchi Y, Kobayashi Y, Arakawa H, Suzuki M, Tamra K, Morikawa A. Polymorphisms in interleukin-4-related genes in patients with minimal change nephrotic syndrome. Pediatr Nephrol. 2009 Mar;24(3):489-95
227. Lai KW, Wei CL, Tan LK, Tan PH, Chiang GS, Lee CG, Jordan SC, Yap HK. Overexpression of interleukin-13 induces minimal-change-like nephropathy in rats. J Am Soc Nephrol. 2007 May;18(5):1476-85
228. Michael AF, Blau E, Vernier RL. Glomerular polyanion. Alteration in aminonucleoside nephrosis. Lab Invest. 1970 Dec;23(6):649-57
229. Chugh SS, Clement LC, Macé C. New insights into human minimal change disease: lessons from animal models. Am J Kidney Dis. 2012 Feb;59(2):284-92
230. Clement LC, Avila-Casado C, Macé C, Soria E, Bakker WW, Kersten S, Chugh SS. Podocyte-secreted angiopoietin-like-4 mediates proteinuria in glucocorticoid-sensitive nephrotic syndrome. Nat Med. 2011 Jan;17(1):117-22
231. Croxtall JD, van Hal PT, Choudhury Q, Gilroy DW, Flower RJ. Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. Br J Pharmacol. 2002 Jan;135(2):511-9
232. Lu NZ, Cidlowski JA. The origin and functions of multiple human glucocorticoid receptor isoforms. Ann N Y Acad Sci. 2004 Jun;1024:102-23. Review
233. De Iudicibus S, Franca R, Martelossi S, Ventura A, Decorti G. Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease. World J Gastroenterol. 2011 Mar 7;17(9):1095-108
234. Mwinyi J, Wenger C, Eloranta JJ, Kullak-Ublick GA. Glucocorticoid

receptor gene haplotype structure and steroid therapy outcome in IBD patients. World J Gastroenterol. 2010 Aug 21;16(31):3888-96
235. Ouyang J, Chen P, Jiang T, Chen Y, Li J. Nuclear HSP90 regulates the glucocorticoid responsiveness of PBMCs in patients with idiopathic nephritic syndrome. Int Immunopharmacol. 2012 Nov;14(3):334-40
236. van Rossum EF, Lamberts SW. Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. Recent Prog Horm Res. 2004; 59:333-57
237. Gross KL, Lu NZ, Cidlowski JA. Molecular mechanisms regulating glucocorticoid sensitivity and resistance. Mol Cell Endocrinol. 2009 Mar 5;300(1-2):7-16
238. Lan HY, Yang N, Nikolic-Paterson DJ, Yu XQ, Mu W, Isbel NM, Metz CN, Bucala R, Atkins RC. Expression of macrophage migration inhibitory factor in human glomerulonephritis. Kidney Int. 2000 Feb;57(2):499-509
239. Berdeli A, Mir S, Ozkayin N, Serdaroglu E, Tabel Y, Cura A. Association of macrophage migration inhibitory factor -173C allele polymorphism with steroid resistance in children with nephrotic syndrome. Pediatr Nephrol. 2005 20(11):1566-71
240. Vivarelli M, D'Urbano LE, Stringini G, Ghiggeri GM, Caridi G, Donn R, Tozzi A, Emma F, De Benedetti F. Association of the macrophage migration inhibitory factor -173*C allele with childhood nephrotic syndrome. Pediatr Nephrol. 2008 May;23(5):743-8
241. Stosic-Grujicic S, Stojanovic I, Nicoletti F. MIF in autoimmunity and novel therapeutic approaches. Autoimmun Rev. 2009 Jan;8(3):244-9
242. Bucala R. MIF, MIF alleles, and prospects for therapeutic intervention in autoimmunity. J Clin Immunol. 2013 Jan;33 Suppl 1:S72-8
243. Tantisira KG, Lasky-Su J, Harada M, Murphy A, Litonjua AA, Himes BE, Lange C, Lazarus R, Sylvia J, Klanderman B, Duan QL, Qiu W, Hirota T, Martinez FD, Mauger D, Sorkness C, Szefler S, Lazarus SC, Lemanske RF Jr, Peters SP, Lima JJ, Nakamura Y, Tamari M, Weiss ST. Genomewide association between GLCCI1 and response to glucocorticoid therapy in asthma. N Engl J Med. 2011 Sep 29;365(13):1173-83
244. Nishibori Y, Katayama K, Parikka M, Oddsson A, Nukui M, Hultenby K, Wernerson A, He B, Ebarasi L, Raschperger E, Norlin J, Uhlén M, Patrakka J, Betsholtz C, Tryggvason K. Glcci1 deficiency leads to proteinuria. J Am Soc Nephrol. 2011 Nov;22(11):2037-46
245. Cheong HI, Kang HG, Schlondorff J. GLCCI1 single nucleotide polymorphisms in pediatric nephrotic syndrome. Pediatr Nephrol. 2012 Sep;27(9):1595-9
246. Wasilewska AM, Zoch-Zwierz WM, Pietruczuk M. Expression of P-glycoprotein in lymphocytes of children with nephrotic syndrome treated with glucocorticoids. Eur J Pediatr. 2006 Dec;165(12):839-44
247. Wasilewska A, Zoch-Zwierz W, Pietruczuk M, Zalewski G. Expression of

P-glycoprotein in lymphocytes from children with nephrotic syndrome, depending on their steroid response. Pediatr Nephrol. 2006 Sep;21(9):1274-80
248. Wasilewska A, Zalewski G, Chyczewski L, Zoch-Zwierz W. MDR-1 gene polymorphisms and clinical course of steroid-responsive nephrotic syndrome in children. Pediatr Nephrol. 2007 Jan;22(1):44-51
249. Choi HJ, Cho HY, Ro H, Lee SH, Han KH, Lee H, Kang HG, Ha IS, Choi Y, Cheong HI. Polymorphisms of the MDR1 and MIF genes in children with nephrotic syndrome. Pediatr Nephrol. 2011 Nov;26(11):1981-8
250. Jafar T, Prasad N, Agarwal V, Mahdi A, Gupta A, Sharma RK, Negi MP, Agrawal S. MDR-1 gene polymorphisms in steroid-responsive versus steroid-resistant nephrotic syndrome in children. Nephrol Dial Transplant. 2011 Dec;26(12):3968-74
251. Chiou YH, Wang LY, Wang TH, Huang SP. Genetic polymorphisms influence the steroid treatment of children with idiopathic nephrotic syndrome. Pediatr Nephrol. 2012 Sep;27(9):1511-7
252. Griffiths AJF, Miller JH, Suzuki DT, et al. Introduction to Genetic Analysis. 7th edition. New York: W. H. Freeman; 2000
253. Yates A, Akanni W, Amode MR, Barrell D, Billis K, Carvalho-Silva D, Cummins C, Clapham P, Fitzgerald S, Gil L, Girón CG, Gordon L, Hourlier T, Hunt SE, Janacek SH, Johnson N, Juettemann T, Keenan S, Lavidas I, Martin FJ, Maurel T, McLaren W, Murphy DN, Nag R, Nuhn M, Parker A, Patricio M, Pignatelli M, Rahtz M, Riat HS, Sheppard D, Taylor K, Thormann A, Vullo A, Wilder SP, Zadissa A, Birney E, Harrow J, Muffato M, Perry E, Ruffier M, Spudich G, Trevanion SJ, Cunningham F, Aken BL, Zerbino DR, Flicek P. Ensembl 2016. Nucleic Acids Res. 2016 Jan 4;44(D1):D710-6
254. Sheffield VC, Weber JL, Buetow KH, Murray JC, Even DA, Wiles K, Gastier JM, Pulido JC, Yandava C, Sunden SL, et al. A collection of tri- and tetranucleotide repeat markers used to generate high quality, high resolution human genome-wide linkage maps. Hum Mol Genet. 1995 Oct;4(10):1837-44
255. Risch N, Merikangas K. The future of genetic studies of complex human diseases. Science. 1996 Sep 13;273(5281):1516-7
256. Collins FS, Guyer MS, Charkravarti A. Variations on a theme: cataloging human DNA sequence variation. Science. 1997 Nov 28;278(5343):1580-1
257. Wang DG, Fan JB, Siao CJ, Berno A, Young P, Sapolsky R, Ghandour G, Perkins N, Winchester E, Spencer J, Kruglyak L, Stein L, Hsie L, Topaloglou T, Hubbell E, Robinson E, Mittmann M, Morris MS, Shen N, Kilburn D, Rioux J, Nusbaum C, Rozen S, Hudson TJ, Lipshutz R, Chee M, Lander ES. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science. 1998 May 15;280(5366):1077-82

258. Kestilä M, Männikkö M, Holmberg C, Gyapay G, Weissenbach J, Savolainen ER, Peltonen L, Tryggvason K. Congenital nephrotic syndrome of the Finnish type maps to the long arm of chromosome 19. Am J Hum Genet. 1994 May;54(5):757-64
259. Ott J, Wang J, Leal SM. Genetic linkage analysis in the age of whole-genome sequencing. Nat Rev Genet. 2015 May;16(5):275-84
260. Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, Klemm A, Flicek P, Manolio T, Hindorff L, Parkinson H. The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. Nucleic Acids Res. 2014 Jan;42(Database issue):D1001-6
261. Hindorff LA, MacArthur J (European Bioinformatics Institute), Morales J (European Bioinformatics Institute), Junkins HA, Hall PN, Klemm AK, and Manolio TA. A Catalog of Published Genome-Wide Association Studies. Available at:www.genome.gov/gwastudies. Accessed 2.6.2016
262. Freedman ML, Monteiro AN, Gayther SA, Coetzee GA, Risch A, Plass C, Casey G, De Biasi M, Carlson C, Duggan D, James M, Liu P, Tichelaar JW, Vikis HG, You M, Mills IG. Principles for the post-GWAS functional characterization of cancer risk loci. Nat Genet. 2011 Jun;43(6):513-8
263. Blattler A, Yao L, Witt H, Guo Y, Nicolet CM, Berman BP, Farnham PJ. Global loss of DNA methylation uncovers intronic enhancers in genes showing expression changes. Genome Biol. 2014 Sep 20;15(9):469
264. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977 Dec;74(12):5463-7
265. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K *et al.*; International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature. 2001 Feb 15;409(6822):860-921
266. Ronaghi M, Uhlén M, Nyrén P. A sequencing method based on real-time pyrophosphate. Science. 1998 Jul 17;281(5375):363-365
267. Shendure J, Porreca GJ, Reppas NB, Lin X, McCutcheon JP, Rosenbaum AM, Wang MD, Zhang K, Mitra RD, Church GM. Accurate multiplex polony sequencing of an evolved bacterial genome. Science. 2005 Sep 9;309(5741):1728-32
268. Rothberg JM, Hinz W, Rearick TM, Schultz J, Mileski W, Davey M, Leamon JH, Johnson K, Milgrew MJ, Edwards M, Hoon J, Simons JF, Marran D, Myers JW, Davidson JF, Branting A, Nobile JR, Puc BP, Light D, Clark TA, Huber M, Branciforte JT, Stoner IB, Cawley SE, Lyons M, Fu Y, Homer N, Sedova M, Miao X, Reed B, Sabina J, Feierstein E, Schorn M, Alanjary M, Dimalanta E, Dressman D, Kasinskas R, Sokolsky T, Fidanza JA, Namsaraev E, McKernan KJ,

Williams A, Roth GT, Bustillo J. An integrated semiconductor device enabling non-optical genome sequencing. Nature. 2011 Jul 20;475(7356):348-52

269. Muzzey D, Evans EA, Lieber C. Understanding the Basics of NGS: From Mechanism to Variant Calling. Curr Genet Med Rep. 2015;3(4):158-165. Epub 2015 Sep 4. Review

270. Bell CJ, Dinwiddie DL, Miller NA, Hateley SL, Ganusova EE, Mudge J, Langley RJ, Zhang L, Lee CC, Schilkey FD, Sheth V, Woodward JE, Peckham HE, Schroth GP, Kim RW, Kingsmore SF. Carrier testing for severe childhood recessive diseases by next-generation sequencing. Sci Transl Med. 2011 Jan 12;3(65):65ra4

271. Fan JB, Chen J, April CS, Fisher JS, Klotzle B, Bibikova M, Kaper F, Ronaghi M, Linnarsson S, Ota T, Chien J, Laurent LC, Loring JF, Nisperos SV, Chen GY, Zhong JF. Highly parallel genome-wide expression analysis of single mammalian cells. PLoS One. 2012;7(2):e30794

272. Yang Y, Mao B, Wang L, Mao L, Zhou A, Cao J, Hu J, Zhou Y, Pan Y, Wei X, Yang S, Mu F, Liu Z. Targeted next generation sequencing reveals a novel intragenic deletion of the LAMA2 gene in a patient with congenital muscular dystrophy. Mol Med Rep. 2015 May;11(5):3687-93

273. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, Liu CL, Neal JW, Wakelee HA, Merritt RE, Shrager JB, Loo BW Jr, Alizadeh AA, Diehn M. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014 May;20(5):548-54

274. Wei CL, Cheung W, Heng CK, Arty N, Chong SS, Lee BW, Puah KL, Yap HK (2005) Interleukin-13 genetic polymorphisms in Singapore Chinese children correlate with long-term outcome of minimal-change disease. Nephrol Dial Transplant 2005 20(4):728-34

275. Alasehirli B, Balat A, Barlas O, Kont A. Nitric oxide synthase gene polymorphisms in children with minimal change nephrotic syndrome. Pediatr Int 2009 51(1):75-8

276. Youssef DM, Attia TA, El-Shal AS, Abduelometty FA. Multi-drug resistance-1 gene polymorphisms in nephrotic syndrome: impact on susceptibility and response to steroids. Gene 2013; 10;530(2):201-7

277. Cizmarikova M, Podracka L, Klimcakova L, Habalova V, Boor A, Mojzis J, Mirossay L. MDR1 Polymorphisms and Idiopathic Nephritic Syndrome in Slovak Children: Preliminary Results. Med Sci Monit. 2015; 21:59-68S

278. Skrzypczyk P, Panczyk-Tomaszewska M, Roszkowska-Blaim M, Wawer Z, Bienias B, Zajgzkowska M, Kilis-Pstrusinska K, Jakubowska A, Szczepaniak M, Pawlak-Bratkowska M, Tkaczyk M. Long-term outcomes in idiopathic nephrotic syndrome: from childhood to adulthood. Clin Nephrol. 2014 Mar;81(3):166-73

279. Trompeter RS, Lloyd BW, Hicks J, White RH, Cameron JS. Long-term outcome for children with minimal-change nephrotic syndrome. Lancet. 1985 Feb 16;1(8425):368-70
280. Fakhouri F, Bocquet N, Taupin P, Presne C, Gagnadoux MF, Landais P, Lesavre P, Chauveau D, Knebelmann B, Broyer M, Grünfeld JP, Niaudet P. Steroid-sensitive nephrotic syndrome: from childhood to adulthood. Am J Kidney Dis. 2003 Mar;41(3):550-7
281. Meadow SR, Sarsfield JK, Scott DG, Rajah SM. Steroid-responsive nephrotic syndrome and allergy: immunological studies. Arch Dis Child. 1981 Jul;56(7):517-24
282. Yap HK, Cheung W, Murugasu B, Sim SK, Seah CC, Jordan SC. Th1 and Th2 cytokine mRNA profiles in childhood nephrotic syndrome: evidence for increased IL-13 mRNA expression in relapse. J Am Soc Nephrol. 1999 Mar;10(3):529-37
283. Abdel-Hafez M, Shimada M, Lee PY, Johnson RJ, Garin EH. Idiopathic nephrotic syndrome and atopy: is there a common link? Am J Kidney Dis. 2009 Nov;54(5):945-53
284. Ruf RG, Fuchshuber A, Karle SM, Lemainque A, Huck K, Wienker T, Otto E, Hildebrandt F. Identification of the first gene locus (SSNS1) for steroid-sensitive nephrotic syndrome on chromosome 2p. J Am Soc Nephrol. 2003 Jul;14(7):1897-900
285. Landau D, Oved T, Geiger D, Abizov L, Shalev H, Parvari R. Familial steroid-sensitive nephrotic syndrome in Southern Israel: clinical and genetic observations. Pediatr Nephrol. 2007 May;22(5):661-9. Epub 2007 Jan 12. Erratum in: Pediatr Nephrol. 2007 May;22(5):761
286. Motoyama O, Sugawara H, Hatano M, Fujisawa T, Iitaka K. Steroid-sensitive nephrotic syndrome in two families. Clin Exp Nephrol. 2009 Apr;13(2):170-3
287. Sümegi V, Haszon I, Bereczki C, Papp F, Túri S. Long-term follow-up after cyclophosphamide and cyclosporine-A therapy in steroid-dependent and -resistant nephrotic syndrome. Pediatr Nephrol. 2008 Jul;23(7):1085-92
288. Kyrieleis HA, Löwik MM, Pronk I, Cruysberg HR, Kremer JA, Oyen WJ, van den Heuvel BL, Wetzels JF, Levtchenko EN. Long-term outcome of biopsy-proven, frequently relapsing minimal-change nephrotic syndrome in children. Clin J Am Soc Nephrol. 2009 Oct;4(10):1593-600
289. Gbadegesin R, Hinkes B, Vlangos C, Mucha B, Liu J, Hopcian J, Hildebrandt F. Mutational analysis of NPHS2 and WT1 in frequently relapsing and steroid-dependent nephrotic syndrome. Pediatr Nephrol. 2007 Apr;22(4):509-13
290. Tsukaguchi H, Sudhakar A, Le TC, Nguyen T, Yao J, Schwimmer JA, Schachter AD, Poch E, Abreu PF, Appel GB, Pereira AB, Kalluri R, Pollak MR. NPHS2 mutations in late-onset focal segmental glomerulosclerosis: R229Q is a common disease-associated allele. J

Clin Invest. 2002 Dec;110(11):1659-66
291. Pereira AC, Pereira AB, Mota GF, Cunha RS, Herkenhoff FL, Pollak MR, Mill JG, Krieger JE. NPHS2 R229Q functional variant is associated with microalbuminuria in the general population. Kidney Int. 2004 Mar;65(3):1026-30
292. Karle SM, Uetz B, Ronner V, Glaeser L, Hildebrandt F, Fuchshuber A. Novel mutations in NPHS2 detected in both familial and sporadic steroid-resistant nephrotic syndrome. J Am Soc Nephrol. 2002 Feb;13(2):388-93
293. Franceschini N, North KE, Kopp JB, McKenzie L, Winkler C. NPHS2 gene, nephrotic syndrome and focal segmental glomerulosclerosis: a HuGE review. Genet Med 2006 Feb;8(2):63-75
294. Hinkes B, Vlangos C, Heeringa S, Mucha B, Gbadegesin R, Liu J, Hasselbacher K, Ozaltin F, Hildebrandt F; APN Study Group. Specific podocin mutations correlate with age of onset in steroid-resistant nephrotic syndrome. J Am Soc Nephrol 2008 19(2):365-71
295. Yoshida K, Shimizugawa T, Ono M, Furukawa H. Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. J Lipid Re. 2002 43(11):1770-2
296. Smart-Halajko MC, Robciuc MR, Cooper JA, Jauhiainen M, Kumari M, Kivimaki M, Khaw KT, Boekholdt SM, Wareham NJ, Gaunt TR, Day IN, Braund PS, Nelson CP, Hall AS, Samani NJ, Humphries SE, Ehnholm C, Talmud PJ. The relationship between plasma angiopoietin-like protein 4 levels, angiopoietin-like protein 4 genotype, and coronary heart disease risk. Arterioscler Thromb Vasc Biol. 2010 Nov;30(11):2277-82
297. Clement LC, Macé C, Avila-Casado C, Joles JA, Kersten S, Chugh SS. Circulating angiopoietin-like 4 links proteinuria with hypertriglyceridemia in nephritic syndrome. Nat Med. 2014; 20(1):37-46
298. Kim RB, Leake BF, Choo EF, Dresser GK, Kubba SV, Schwarz UI, Taylor A, Xie HG, McKinsey J, Zhou S, Lan LB, Schuetz JD, Schuetz EG, Wilkinson GR. Identification of functionally variant MDR1 alleles among European Americans and African Americans. Clin Pharmacol Ther. 2001; 70(2):189-99
299. Anglicheau D, Flamant M, Schlageter MH, Martinez F, Cassinat B, Beaune P, Legendre C, Thervet E. Pharmacokinetic interaction between corticosteroids and tacrolimus after renal transplantation. Nephrol Dial Transplant. 2003; 18(11):2409-14
300. Ameyaw MM, Regateiro F, Li T, Liu X, Tariq M, Mobarek A, Thornton N, Folayan GO, Githang'a J, Indalo A, Ofori-Adjei D, Price-Evans DA, McLeod HL. MDR1 pharmacogenetics: frequency of the C3435T mutation in exon 26 is significantly influenced by ethnicity. Pharmacogenetics. 2001; 11(3):217-21
301. Zalewski G, Wasilewska A, Zoch-Zwierz W, Chyczewski L. Response to

prednisone in relation to NR3C1 intron B polymorphisms in childhood nephrotic syndrome. Pediatr Nephrol. 2008 Jul;23(7):1073-8
302. Teeninga N, Kist-van Holthe JE, van den Akker EL, Kersten MC, Boersma E, Krabbe HG, Knoers NV, van der Heijden AJ, Koper JW, Nauta J. Genetic and in vivo determinants of glucocorticoid sensitivity in relation to clinical outcome of childhood nephrotic syndrome. Kidney Int. 2014 Jun;85(6):1444-53
303. Bouchireb K, Boyer O, Gribouval O, Nevo F, Huynh-Cong E, Morinière V, Campait R, Ars E, Brackman D, Dantal J, Eckart P, Gigante M, Lipska BS, Liutkus A, Megarbane A, Mohsin N, Ozaltin F, Saleem MA, Schaefer F, Soulami K, Torra R, Garcelon N, Mollet G, Dahan K, Antignac C. NPHS2 mutations in steroid-resistant nephrotic syndrome: a mutation update and the associated phenotypic spectrum. Hum Mutat. 2014 Feb;35(2):178-86
304. Tryggvason K, Patrakka J, Wartiovaara J. Hereditary proteinuria syndromes and mechanisms of proteinuria. N Engl J Med. 2006 Mar 30;354(13):1387-401. Review
305. Wiggins RC. The spectrum of podocytopathies: a unifying view of glomerular diseases. Kidney Int. 2007 Jun;71(12):1205-14. Review
306. Berdeli A, Mir S, Yavascan O, Serdaroglu E, Bak M, Aksu N, Oner A, Anarat A, Donmez O, Yildiz N, Sever L, Tabel Y, Dusunsel R, Sonmez F, Cakar N. NPHS2 (podicin) mutations in Turkish children with idiopathic nephrotic syndrome. Pediatr Nephrol. 2007 Dec;22(12):2031-40
307. Köttgen A, Hsu CC, Coresh J, Shuldiner AR, Berthier-Schaad Y, Gambhir TR, Smith MW, Boerwinkle E, Kao WH. The association of podocin R229Q polymorphism with increased albuminuria or reduced estimated GFR in a large population-based sample of US adults. Am J Kidney Dis. 2008 Nov;52(5):868-75
308. Schwarz K, Simons M, Reiser J, Saleem MA, Faul C, Kriz W, Shaw AS, Holzman LB, Mundel P. Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. J Clin Invest. 2001 Dec;108(11):1621-9 Gee HY, Sadowski CE, Aggarwal PK, Porath JD, Yakulov TA, Schueler M, Lovric S, Ashraf S, Braun DA, Halbritter J, Fang H, Airik R, Vega-Warner V, Cho KJ, Chan TA, Morris LG, Ffrench-Constant C, Allen N, McNeill H, Büscher R, Kyrieleis H, Wallot M, Gaspert A, Kistler T, Milford DV, Saleem MA, Keng WT, Alexander SI, Valentini RP, Licht C, Teh JC, Bogdanovic R, Koziell A, Bierzynska A, Soliman NA, Otto EA, Lifton RP, Holzman LB, Sibinga NE, Walz G, Tufro A, Hildebrandt F. FAT1 mutations cause a glomerulotubular nephropathy. Nat Commun. 2016 Feb 24;7:10822
309. Yaddanapudi S, Altintas MM, Kistler AD, Fernandez I, Möller CC, Wei C, Peev V, Flesche JB, Forst AL, Li J, Patrakka J, Xiao Z, Grahammer

F, Schiffer M, Lohmüller T, Reinheckel T, Gu C, Huber TB, Ju W, Bitzer M, Rastaldi MP, Ruiz P, Tryggvason K, Shaw AS, Faul C, Sever S, Reiser J. CD2AP in mouse and human podocytes controls a proteolytic program that regulates cytoskeletal structure and cellular survival. J Clin Invest. 2011 Oct;121(10):3965-80

310. Hyvönen ME, Ihalmo P, Sandholm N, Stavarachi M, Forsblom C, McKnight AJ, Lajer M, Maestroni A, Lewis G, Tarnow L, Maestroni S, Zerbini G, Parving HH, Maxwell AP, Groop PH, Lehtonen S. CD2AP is associated with end-stage renal disease in patients with type 1 diabetes. Acta Diabetol. 2013 Dec;50(6):887-97

311. Goode NP, Shires M, Khan TN, Mooney AF. Expression of alpha-actinin-4 in acquired human nephrotic syndrome: a quantitative immunoelectron microscopy study. Nephrol Dial Transplant. 2004 Apr;19(4):844-51

312. Sterk LM, de Melker AA, Kramer D, Kuikman I, Chand A, Claessen N, Weening JJ, Sonnenberg A. Glomerular extracellular matrix components and integrins. Cell Adhes Commun. 1998 Mar;5(3):177-92

313. Kojima K, Davidovits A, Poczewski H, Langer B, Uchida S, Nagy-Bojarski K, Hovorka A, Sedivy R, Kerjaschki D. Podocyte flattening and disorder of glomerular basement membrane are associated with splitting of dystroglycan-matrix interaction. J Am Soc Nephrol. 2004 Aug;15(8):2079-89

314. Kriz W, Hähnel B, Rösener S, Elger M. Long-term treatment of rats with FGF-2 results in focal segmental glomerulosclerosis. Kidney Int. 1995 Nov;48(5):1435-50

315. Mao J, Zhang Y, Du L, Dai Y, Yang C, Liang L. Expression profile of nephrin, podocin, and CD2AP in Chinese children with MCNS and IgA nephropathy. Pediatr Nephrol. 2006 Nov;21(11):1666-75

316. Lenkkeri U, Männikkö M, McCready P et al. Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations. Am J Hum Genet. 1999; 64: 51-61