Department of Bacteriology and Immunology
Haartman Institute
Biomedicum Helsinki
University of Helsinki
Finland

NEPHRIN IN DIABETES AND IN DIABETES-RELATED CONDITIONS

Emphasis on urinary proteins immunoreactive with nephrin antibodies

ANU PÄTÄRI

ACADEMIC DISSERTATION

To be presented for public discussion, with the permission of the Medical Faculty of the University of Helsinki, in the Lecture Hall 2 in Biomedicum, Haartmaninkatu 8, Helsinki, on September 17th, 2005, at 12 noon.

HELSINKI 2005

SUPERVISED BY

Harry Holthöfer, M.D., Ph.D.
Docent
Department of Bacteriology and Immunology
Haartman Institute
University of Helsinki
Finland

REVIEWED BY

Ulf-Håkan Stenman, M.D., Ph.D.
Professor
Department of Clinical Chemistry
University of Helsinki
Finland

and

Arno Hänninen, M.D., Ph.D.
Docent
Department of Medical Microbiology
MediCity Research Laboratory
University of Turku
Turku

OFFICIAL OPPONENT

Carola Grönhagen-Riska, M.D., Ph.D.
Professor
Department of Medicine
Division of Nephrology
Helsinki University Central Hospital and
University of Helsinki
Helsinki

ISBN 952-91-9146-4 (paperback)
ISBN 952-10-2642-1 (pdf)
http://ethesis.helsinki.fi

Yliopistopaino
Helsinki 2005

Science is a balance between faith and criticism

Too much faith
- you go wrong
Too much criticism
- you go nowhere

The author

To Timo and my family

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on four original publications, which are referred to in the text by their Roman numerals. In addition, some unpublished data are included.

I Z Cheng*, A Pätäri*, K Aalto-Setälä, D Novikov, D Schlöndorff and H Holthöfer: Hypercholesterolaemia is a prerequisite for puromycin inducible damage in mouse kidney. Kidney International, 63:107-12, 2003.

II A Pätäri, C Forsblom, M Havana, H Taipale, P-H Groop, H Holthöfer and the FinnDiane Study Group: Nephrinuria in diabetic nephropathy of type 1 diabetes. Diabetes, 52:2969-74, 2003.

III A Pätäri, C Forsblom, P-H Groop, H Holthöfer, and the FinnDiane Study Group: The 75 kDa urinary nephrin may serve as a protective marker for diabetic nephropathy in a follow-up study of type 1 diabetic patients. Submitted.

IV A Pätäri*, P Karhapää*, H Taipale, U Salmenniemi, E Ruotsalainen, P Vanninen, H Holthöfer and M Laakso: A 100 kDa urinary protein associates with insulin resistance in offspring of type 2 diabetic patients. Diabetologia, in press.

*These two authors contributed equally to the study

ABBREVIATIONS

ACE	Angiotensin converting enzyme
AER	Albumin excretion rate
AGE	Advanced glycation end-product
ApoE	Apolipoprotein E
AUC	Area under curve
Clamp	Euglycemic hyperinsulinemic clamp technique
CNF	Congenital nephrotic syndrome of the Finnish type
DN	Diabetic nephropathy
EM	Electron microscopy
ESRD	End-stage renal disease
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
4-HNE	4-hydroxynonenal
IVGTT	Intravenous glucose tolerance test
LDL	Low density lipoprotein
Macro	Macroalbuminuric patients
MDA	Malonyldialdehyde
M/I	Whole body glucose uptake
Micro	Microalbuminuric patients
Normo	Normoalbuminuric patients
OGTT	Oral glucose tolerance test
PAN	Puromycin aminonucleoside nephrosis
PKC	Protein kinase C
RAGE	Receptor for advanced glycation end products
RAS	Renin-angiotensin system
ROS	Reactive oxygen species/radicals
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor

1. ABSTRACT

The number of diabetic patients is an increasing worldwide health care problem. Approximately one third will eventually develop the diabetic kidney complication, diabetic nephropathy. Microalbuminuria is the most widely used marker but at the time of diagnosis there are already advanced lesions in the kidney filtration apparatus, the glomeruli. Nephrin is an important molecule in the glomeruli and it forms part of the filtration barrier, through which the primary urine is filtered. The expression of nephrin shows characteristic changes in diabetes and in other acquired proteinuric diseases.

Hypercholesterolemia is one of the known risk factors for kidney damage and a constant finding in kidney diseases. The present study investigated the causal relationship of hypercholesterolemia and proteinuria, and the effect of hypercholesterolemia on glomerular damage and on nephrin expression in the mouse. The study found that hypercholesterolemia was a prerequisite for proteinuria and that nephrin expression was diminished both at the mRNA and protein levels. Increased lipid peroxidation was involved in the pathogenic process in this model.

In the development of diabetic nephropathy, nephrin expression increases initially just before albuminuria starts and diminishes at the stage of overt albuminuria. In the present study, type 1 diabetic patients with or without nephropathy were studied for the presence of urinary proteins detectable with nephrin antisera. First, urine from one third of the patients showed proteins that reacted with nephrin antisera. The presence of these protein fragments was not associated with clinical variables. Second, the 75 kDa protein turned out to be the most specific for nephrin. In two separate type 1 diabetic patient cohorts the occurrence of this 75 kDa nephrin was significantly lower in patients with more severe nephropathy, and the occurrence was highest in the diabetic patients with no clinical signs of nephropathy. Of type 1 diabetic patients 73 were followed for an average of 7.8 years for the progression of nephropathy. 20% of progressors and 42% of non-progressors showed 75 kDa nephrin in urine at baseline ($p=0.23$). Further studies are needed to evaluate whether this protein may serve as a marker for progression of diabetic nephropathy. In this cohort, healthy controls were negative for the presence of urinary proteins reacting with nephrin antiserum.

Nondiabetic first-degree relatives of T2DM patients have an almost threefold increased lifetime risk of diabetes compared to the background population. Type 2 diabetes is often preceded by a stage characterized by alterations in glucose metabolism. First-degree relatives of type 2 diabetic patients are more insulin resistant, and they may also show other signs of the metabolic syndrome, such as central adiposity, hypertension, glucose intolerance, hypercoagulability, microalbuminuria, and dyslipidemia. In the present study urine samples from the offspring of type 2 diabetic patients were investigated for the presence of proteins reacting with nephrin antiserum. Of the offspring, 27% showed a 100 kDa urinary protein in the urine, while healthy controls were all negative. The offspring were further divided into strongly positive, weakly positive and negative groups according to the presence of this protein. The strongly positive offspring were significantly

more insulin resistant compared to the negative offspring and their nonoxidative glucose disposal was lower. It is possible that insulin resistance and diabetes cause changes in podocyte metabolism and in nephrin expression, which is reflected in urine.

2. REVIEW OF THE LITERATURE

2.1. The kidney; filtration function and structure

The main functions of the kidneys are secretion of metabolic end products, maintenance of correct fluid, electrolyte, and acid-base balance of the body and participation in production of crucial substances like the vitamin D and erythropoietin (Guyton, 1991). One kidney (Figure 2.1) contains an estimated 500 000 nephrons which are the basic functional units forming urine. A nephron (Figure 2.2) is composed of a glomerulus (the capillary bundle), through which fluid is filtered from blood and primary urine is produced, and a long tubule in which the primary urine is transformed into final urine. The tubule can be divided anatomically and functionally into distinct parts with specific roles in water and electrolyte balance, pH regulation, reabsorption of filtered substances and secretion of metabolic end products. From tubules urine flows through the collecting duct system to the renal pelvis, and finally via ureters to the bladder. The outer zone of the kidney, the cortex, contains all the glomeruli and the inner zone, the medulla, contains parts of the tubules and the final parts of the collecting ducts. Blood enters the glomerulus via the afferent arteriole and then leaves via the efferent arteriole, which directs the blood then through the peritubular capillary network surrounding the entire tubular system. The tubular epithelial cells are in addition to reabsorbing valuable substances from the tubular lumen also capable of actively secreting substances from the blood into the urine (O'Callaghan and Brenner, 2000).

Figure 2.1

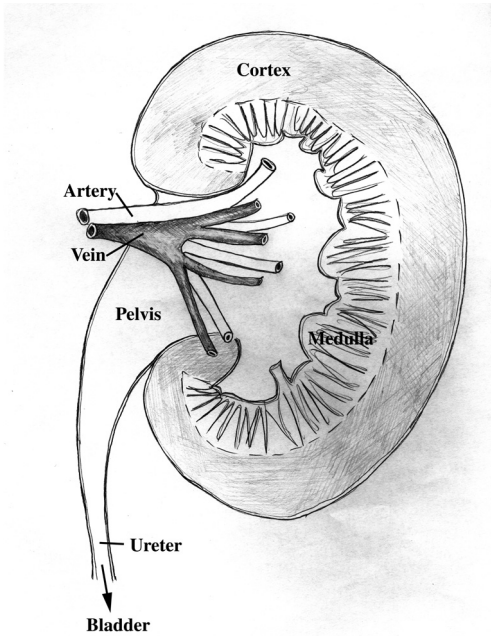
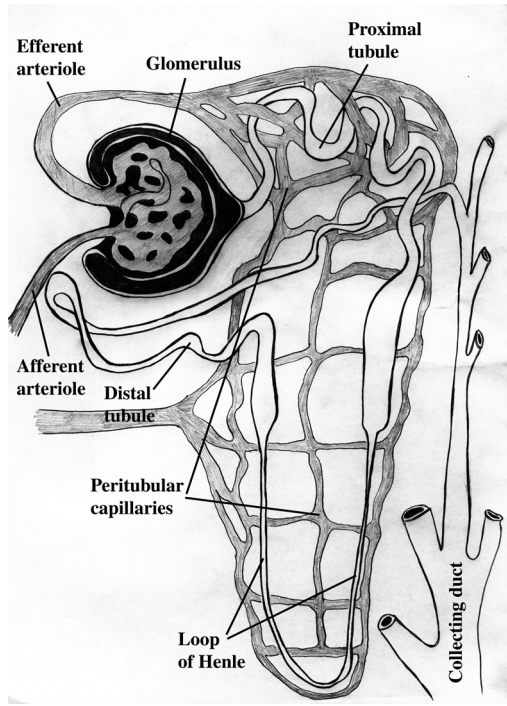
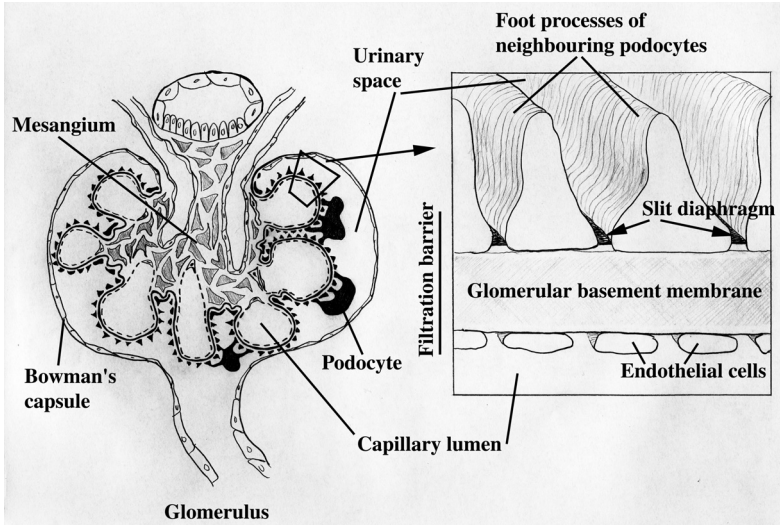


Figure 2.2



An estimated 180 liters of primary urine is filtered each day through the selectively permeable glomerular filtration barrier into the Bowman's capsule (the urinary space) surrounding the glomerulus (Figure 2.3). The filtration barrier itself comprises capillary endothelial cells, glomerular basement membrane (GBM) and visceral epithelial cells, called podocytes. The capillary endothelial cells have abundant 70-100 nm openings (fenestrations), which are aligned with negatively charged glycoproteins and lipids (Tisher and Madsen, 1991). This unique porosity allows free contact of components of the blood circulation with the underlying GBM, although favoring filtration of cationic molecules. The negatively charged, 300 nm-thick, GBM is composed mainly of type IV collagen and laminin, as well as heparan sulphate proteoglycans (agrin and perlecan), fibronectin, and nidogen (Miner, 1999; Timpl, 1989). The podocytes are facing directly the urinary space. They have long projections from which the primary and secondary foot processes arise, and attach to the urinary side of the glomerular basement membrane. The foot processes from neighbouring podocytes interdigitate and it is proposed that they form form 35-45 nm zipper-like filtration slit diaphragms separating foot processes from each other (Rodewald and Karnovsky, 1974; Tryggvason, 1999). This arrangement allows free passage of small molecules through the slit while preventing leakage of large molecules into the primary urine. It has been suggested that the slits may be partially elastic and that the slit width may increase with pulsating intraglomerular pressure (Kriz et al., 1996; Yu et al., 1997). Electron microscopic (EM) studies have shown that the width of the slit might vary even between 20-50 nm (Ohno et al., 1992) although different fixation methods may alter the dimensions measurable by EM (Furukawa et al., 1991). The filtration barrier functions both as a size-selective and a charge-selective sieve. The glomerulus also contains mesangial cells, which provide a scaffold to support the capillary loops and have contractile and phagocytic properties (Hawkins et al., 1990; Pfeilschifter et al., 1993).

Figure 2.3



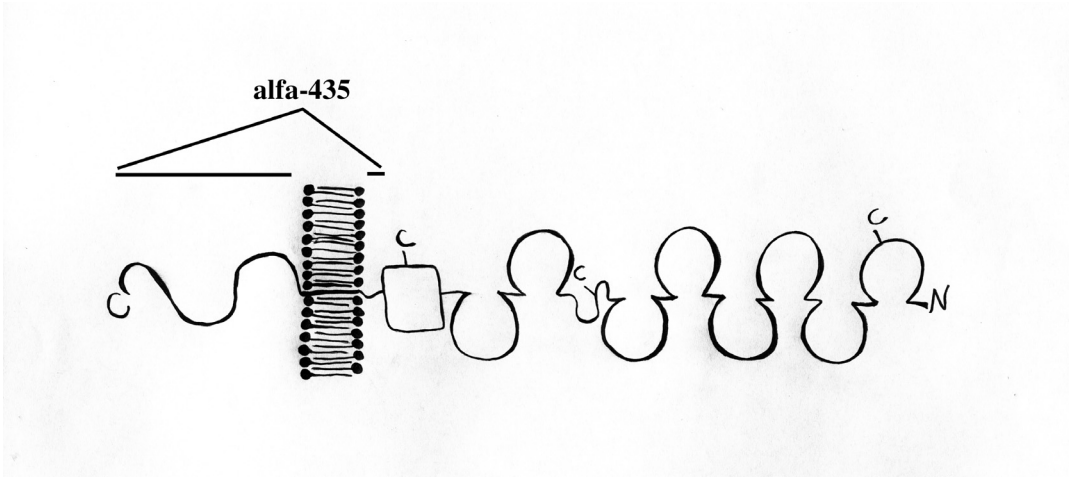
2.2. Nephrin as an interacting component of the podocyte proteome

2.2.1. Nephrin

The glomerular filtration barrier is affected in numerous primary and secondary kidney diseases resulting in leakage of albumin and larger plasma proteins into the urine with generalized oedema and nephrotic syndrome as the final consequence. Congenital nephrotic syndrome of the Finnish type (CNF) is an autosomal, recessive disorder, characterized by massive proteinuria in utero and nephrosis at birth (Hallman et al., 1956; Norio et al., 1964). This syndrome is seen in 1:10000 to 1:8000 newborns in Finland (Holmberg et al., 1996) and serves as a model disease for podocyte-specific proteinuria. The typical clinical symptoms include severe hypoproteinemia due to massive loss of circulating proteins into the urine most likely due to a filtration slit defect. Other symptoms include edema, hyperlipidemia, and susceptibility for thromboembolic complications and for bacterial infections. The patients show overt proteinuria of intrauterine onset, which is associated with enlargement of the placenta and high alpha-fetoprotein levels in amnionic fluid and in maternal serum (Holmberg et al., 1996). The characteristic pathologic findings are fusion of the podocyte foot processes (foot process effacement), dilation of the proximal tubules, mesangial hypercellularity, and thickening of the GBM (Hallman et al., 1956; Huttunen et al., 1980; Ljungberg et al., 1993).

Using positional cloning Kestilä et al. were able to identify the nephrin gene (*NPHS1*) mutated in CNF (Kestila et al., 1998). This gene is located in the long arm of chromosome nineteen in locus 13.1 and contains 29 exons (Kestila et al., 1994; Mannikko et al., 1995). The gene product, nephrin, is a 1241-residue transmembrane protein belonging to the immunoglobulin super family (Figure 2.4 and 2.5). Two mutations account for most Finnish patients and lead to synthesis of a truncated form of nephrin; frameshift deletion in exon 2 (Finn major) and nonsense mutation in exon 26 (Finn minor). In other countries point mutations in the nephrin gene cause sporadic cases closely resembling CNF (Beltcheva et al., 2001; Lenkkeri et al., 1999). Although CNF is a recessive disorder, fetal carriers of the nephrin mutation show fusion of the podocyte foot processes, temporary proteinuria, and a false positive alpha-fetoprotein test (Patrakka et al., 2002a). Later on one functional allele is enough and carriers show normal kidney function. Nephrin-deficient mouse models strengthen the crucial role of nephrin in the glomerular filtration function by expressing heavy proteinuria (Putaala et al., 2001) (Hamano et al., 2002; Rantanen et al., 2002). Interestingly, one third of the foot processes were fused in electron micrographs and there was over 60% decrease of nephrin-specific mRNA level in glomeruli of asymptomatic heterozygous nephrin-deficient mice (Rantanen et al., 2002).

Figure 2.4

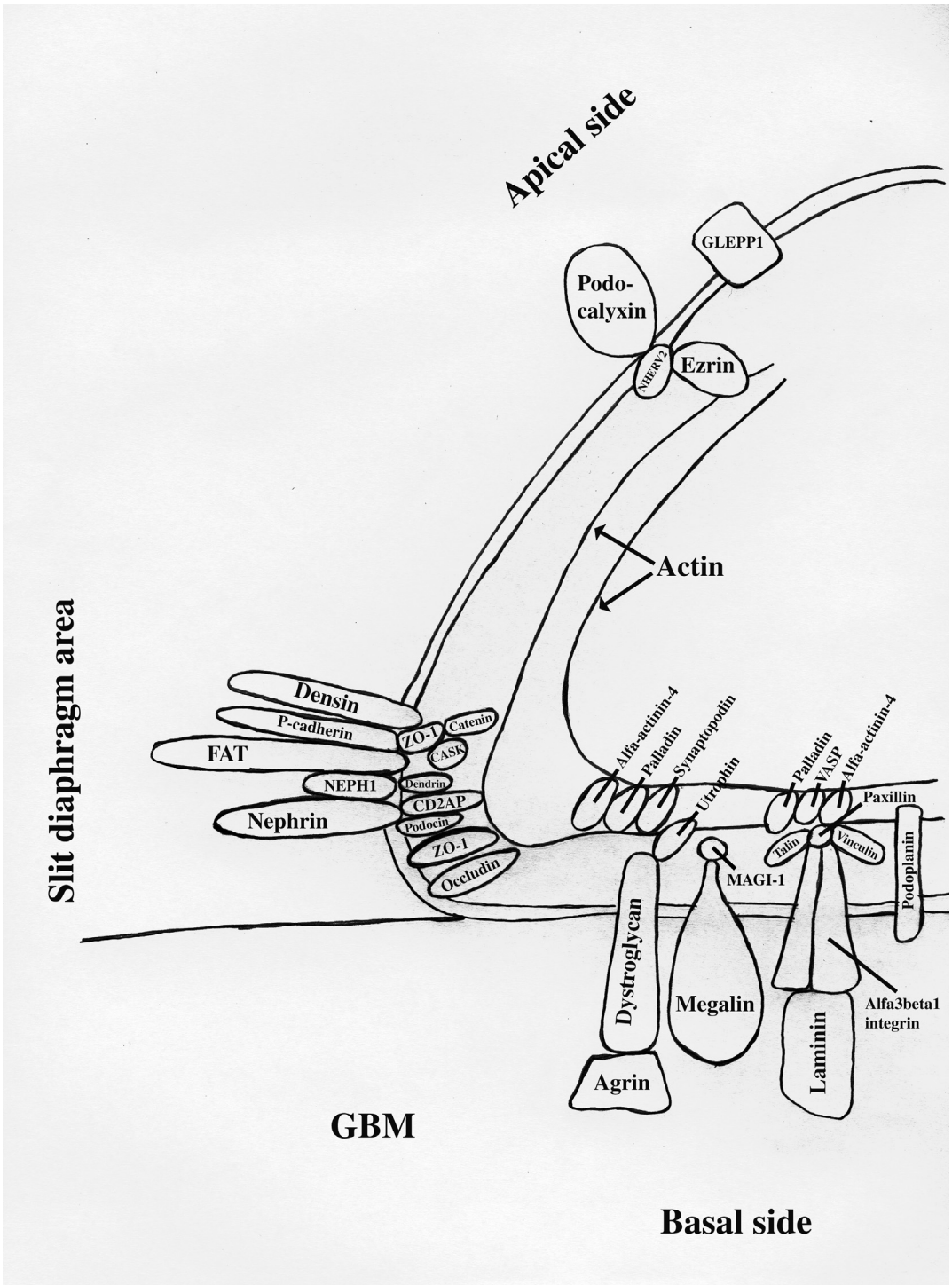


Nephrin is expressed in the islets of Langerhans in the pancreas (Palmen et al., 2001; Putaala et al., 2001). Positive protein staining has been found in the pancreatic beta cells (Palmen et al., 2001), and recently in islet microendothelium (Zanone et al., 2005). The exact function of pancreatic nephrin is still not known, but it may serve as a structural protein in islet microendothelium (Zanone et al., 2005). Moreover, controversial data on whether nephrin is truly expressed in the pancreas do exist suggesting that nephrin has not major significance outside the kidney (Kuusniemi et al., 2004). Nephrin is expressed also in distinct locations in the mouse brain during brain development (Putaala et al., 2001), in the Sertoli cells of mouse testis (Liu et al., 2001), and in rat spleen (Ahola et al., 1999). In the kidney nephrin is specifically located at the slit diaphragm (Holthofer et al., 1999; Ruotsalainen et al., 1999) and its strands contribute to the protein scaffold of the filtration slit as seen in electron tomography (Wartiovaara et al., 2004). Spliced nephrin (nephrin α) has been found at the mRNA level in both the rat and human kidney (Ahola et al., 1999; Holthofer et al., 1999; Luimula et al., 2000a) as well as in the pancreas (Palmen et al., 2001). Nephrin α lacks the whole amino acid sequence spanning the transmembrane domain encoded by exon 24 in the human and thus could represent a soluble form of the protein. The eight extracellular Ig-like domains of nephrin are of type C2 that is typically found in proteins participating in cell-cell (Brummendorf and Rathjen, 1995; Chothia and Jones, 1997) or cell-matrix interactions (Fahrig et al., 1987). Nephrin has three free cysteine residues which are suggested to form disulfide bridges between different nephrin molecules so that homophilic interactions between different nephrin molecules over the slit are possible (Kestila et al., 1998; Tryggvason, 1999). Nephrin was shown to form a homophilic interaction with nephrin and a heterophilic interaction with NEPH1 (Gerke et al., 2003) (Barletta et al., 2003; Liu et al., 2003). The homophilic interaction of extracellular nephrin was of high affinity and was promoted by calcium ions (Khoshnoodi et al., 2003). The 90-kDa NEPH1 is a protein with weak homology

and structural similarity to nephrin. The lack of NEPH1 leads to prenatal lethality with proteinuria in *Neph1* -/- mice (Donoviel et al., 2001). The calculated molecular mass of nephrin is 132.5 kDa, while in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) nephrin runs as a 185-200 kDa protein doublet suggesting posttranslational modifications (Ahola et al., 1999; Topham et al., 1999). In the extracellular part of human nephrin there are ten potential sites for N-glycosylation (Kestila et al., 1998) and it has been shown that mouse nephrin is N-glycosylated (Holzman et al., 1999) and that N-glycosylation of nephrin is critical for its proper folding and localization in the plasma membrane (Yan et al., 2002). Glycosylation is needed also for proper interaction with NEPH1 (Gerke et al., 2003). Nephrin carries seven potential attachment sites for heparan sulfate (Kestila et al., 1998).

Nephrin also contains a fibronectin type III-like domain in the extracellular part near the transmembrane region and an intracellular C-terminal part. Nephrin has signaling functions enabled by the nine tyrosines of the intracellular domain, some of which are phosphorylated during ligand binding as well as endogenously (Verma et al., 2003). Oligomerized nephrin is associated with signalling microdomains, lipid rafts, in a cholesterol dependent manner (Simons et al., 2001). In vivo injection of antibodies against podocyte-specific 9-O-acetylated GD3 ganglioside, which is an important component of lipid rafts, leads to morphological changes of the filtration slits resembling foot process effacement. In this model nephrin dislocated to the apical pole of the narrowed filtration slits and was tyrosine phosphorylated (Simons et al., 2001). Furthermore, clustering of extracellular domain of nephrin by nephrin antibodies in a cell line leads to disruption of cell-cell contacts (Khoshnoodi et al., 2003) and to phosphorylation of nephrin by Src family kinases (Lahdenperä et al., 2003). Similarly intravenous injection of the nephrin-specific monoclonal antibody 5-1-6 induced massive proteinuria in rats (Orikasa et al., 1988) and decreased nephrin expression (Kawachi et al., 2000). Phosphorylated nephrin is able to bind p85 regulatory subunit of phosphoinositide 3-OH kinase (PI3K) and activate by phosphorylation the PI3K target protein, serine-threonine kinase AKT (Huber et al., 2003a). This leads to phosphorylation of downstream molecules, one of which is the proapoptotic Bcl-2 family member Bad. Phosphorylation of Bad prevents detachment-induced apoptosis and safeguards podocyte viability (Huber et al., 2003a). However, Foster et al. suggested that vascular endothelial growth factor (VEGF) treatment caused nephrin phosphorylation together with decrease in AKT-signaling (Foster et al., 2005). Glycoprotein VEGF is a key survival factor for vascular endothelium (Ferrara, 2002). Down-regulation or neutralization of circulating VEGF caused proteinuria with endothelial cell detachment, podocyte changes, and reduction in nephrin expression (Sugimoto et al., 2003). The inflammatory cytokines interleukin-1β and tumor necrosis factor-α are able to up-regulate nephrin expression in podocytes in vitro and this phenomenon involves activity of an unknown protein kinase (Huwiler et al., 2003). The protein kinase C (PKC) pathway may be involved in nephrin signaling (Wang et al., 2001b).

Figure 2.5


Apical side
GLEPP1
Podo-
calyxin
NHERF2
Ezrin
Actin
Slit diaphragm area
Densin
P-cadherin
FAT
NEPH1
Nephrin
ZO-1
Catenin
CASK
Dendrin
CD2AP
Podocin
ZO-1
Occludin
Alfa-actimin-4
Palladin
Synaptopodin
Utrophin
Palladin
VASP
Alfa-actimin-4
Paxillin
Talin
Vinculin
Podoplanin
MAGI-1
Dystroglycan
Megalin
Laminin
Alfa3beta1
integrin
Agrin
GBM
Basal side

2.2.2. Proteins of the slit diaphragm area

CD2 adaptor protein (CD2AP), initially found to be associated with the T-lymphocyte molecule CD2, is also present at the slit membrane level in podocytes and is linked to the intracellular part of nephrin via its C-terminal domain (Palmen et al., 2002; Shih et al., 2001). The N-terminal part of CD2AP binds to p85 and potentiates the nephrin-induced AKT activation (Huber et al., 2003a). CD2AP-knockout mice have defects in the foot processes of podocytes and hyperplasia of the mesangial cells with extracellular matrix depositions (Shih et al., 1999). Although CD2AP knockout mice develop nephrotic syndrome similar to CNF, the symptoms develop later, at the age of 3-4 weeks. This suggests that the function of CD2AP might be compensated for at some stage by other proteins (Shih et al., 1999). Kidneys from CD2AP -/- mice initially exhibited normal nephrin localization, but with aging the foot processes became effaced and the nephrin disappeared (Li et al., 2000). CD2AP is connected directly or indirectly to F-actin (Welsch et al., 2001), and nephrin in the slits is linked to the actin cytoskeleton, possibly through CD2AP or other intermediary linker proteins (Yuan et al., 2002a). These may include densin (Ahola et al., 2003), IQGAP1 (Liu et al., 2005), p120 catenin, P-cadherin, and CASK (Lehtonen et al., 2004), which have very recently been found being directly or indirectly linked to nephrin (Figure 2.5).

Another important protein at the slit area is podocin, which is mutated in autosomal recessive familiar focal segmental glomerulosclerosis, sporadic focal segmental glomerulosclerosis, and in some CNF patients in whom nephrin mutations are not found (Boute et al., 2000; Karle et al., 2002; Koziell et al., 2002; Roselli et al., 2002; Tsukaguchi et al., 2000). Podocin is a hairpin-like integral membrane protein belonging to the stomatin family and it is also accumulated in an oligomerized form in lipid rafts, localizing at the insertion site of the slit diaphragm (Roselli et al., 2002; Schwarz et al., 2001). Pull-down experiments and co-immunoprecipitations have revealed that podocin associates via its C-terminal domain with CD2AP and nephrin, and may serve as a scaffolding protein in the organization of the slit diaphragm complex (Huber et al., 2001; Schwarz et al., 2001). Podocin also increased the ability of nephrin to activate mitogen-activated protein kinase cascades in the embryonic kidney 293T cell system by recruiting nephrin into lipid rafts (Huber et al., 2001; Huber et al., 2003b). Mutations in C-terminal podocin causes retention of both podocin and nephrin in endoplasmic reticulum showing no staining of these proteins at the plasma membrane in transfected human embryonic kidney cells (Nishibori et al., 2004). Depending on the mutation podocin either does not leave the endoplasmic reticulum or localize in lipid rafts on the plasma membrane and is consequently unable to potentiate nephrin signaling (Huber et al., 2003b). Knocking down podocin expression in a podocyte cell line by mRNA interference decreased nephrin expression by 70% and altered nephrin localization from the membrane surface to the nuclear area (Fan et al., 2004). Podocin-deficient mice also show antenatal proteinuria, fusion of foot processes and massive mesangial sclerosis with vastly reduced nephrin expression (Roselli et al., 2004). Podocin interacts with the C-terminal domain of NEPH1 and with two other NEPH-family proteins, NEPH2 and NEPH3, which are similar to nephrin (Ihalmo et al., 2003; Sellin et al., 2003).

Defective action of podocin might have a role in the development of secondary focal segmental glomerulosclerosis observed in various diseases such as diabetic nephropathy, HIV nephropathy and morbid obesity.

The membrane protein ZO-1, namely its isoform lacking motif-alpha, is expressed in the cytoplasmic surface of the slit (Kurihara et al., 1992a; Kurihara et al., 1992b). ZO-1 is not attached to nephrin and the same holds true for the transmembrane protein occludin (Holthofer et al., 1999). ZO-1 has a different staining pattern compared to nephrin (Kawachi et al., 2000) and it is normally found at the cytosolic side of tight junctions where it interacts with occludin and with the actin cytoskeleton (Balda and Matter, 2000). Early in podocyte development tight junctions are found in place of the slit membranes and therefore it was suggested that the mature slit membrane is actually a modified tight junction (Schnabel et al., 1990). Supporting this view, Kawachi et al. reported down-regulation of ZO-1 in proteinuric diseases (Kawachi et al., 1997). Other studies, however, failed to identify any changes in ZO-1 expression during proteinuria (Bains et al., 1997; Rantanen et al., 2002; Yuan et al., 2002b). In addition, other proteins characteristic for tight junctions have not been found from the slit area. Instead, members of adherens junctions, α-, β-, and γ-catenin as well as P-cadherin that can also associate with ZO-1 were observed (Reiser et al., 2000). Since P-cadherin deficient mice and humans with a mutation in P-cadherin gene show no kidney phenotype it may not have significance for the glomerular filtration (Dahl et al., 2002; Radice et al., 1997; Sprecher et al., 2001). Another member of the cadherin super family, FAT, has been localized to the slit area co-localizing with ZO-1 and nephrin but its function still remains unknown (Inoue et al., 2001).

Mutations in a cytosolic actin-filament cross-linking protein, α-actinin-4, have also been shown to cause another form of proteinuric disease, the autosomal dominant familial focal segmental glomerulosclerosis (Kaplan et al., 2000). Most likely this protein is one of the links for the slit diaphragm proteins to the actin cytoskeleton for final functional effects: changing rapidly the shape of podocytes from the well organized orderly foot processes to the flattening found in proteinuric states. An intact submembranous actin cytoskeleton appears to be indispensable for maintaining podocyte architecture. Endlich et al. have shown that mechanical stress induces reorganization of the actin cytoskeleton in podocytes by a calcium and Rho kinase dependent mechanism (Endlich et al., 2001). Saleem et al. also showed that nephrin and podocin expression was altered in a podocyte cell line after treatment with cytochalasin D, an agent known to de-polymerize actin stress fibers (Saleem et al., 2002).

2.2.3. Apical side of podocytes

The apical membrane of foot processes constitutes another functional unit. Podocalyxin is a highly glycosylated integral membrane protein which is thought to contribute to the maintenance of the negative charge in the podocyte plasma membrane and thus keep the filtration pores open (Dekan et al., 1991; Kerjaschki et al., 1984). It is mainly distributed on the apical surface of glomerular podocytes and contributes directly to the stability of foot processes, because a genetic knockout resulted in immature glomeruli with flattened embryonic podocytes (Doyonnas et al., 2001). GLEPP1 is a receptor

tyrosine phosphatase present also on the apical side of the podocytes and it is thought to regulate the glomerular filtration through an effect on podocyte structure and function (Thomas et al., 1994; Wharram et al., 2000). GLEPP1 knockout mice showed reduced nephrin expression, reduced glomerular filtration rate, fewer foot processes, but no detectable increase in proteinuria (Wharram et al., 2000).

2.2.4. Basal side of podocytes

The sole of the foot process is linked to the GBM by dystroglycan (Regele et al., 2000), α3β1 integrin (Kerjaschki et al., 1989), podoplanin (Breiteneder-Geleff et al., 1997; Matsui et al., 1998), and megalin (Kerjaschki and Farquhar, 1983). α3β1 integrin is important for podocyte maturation (Kreidberg et al., 1996) but in glomerular diseases it comprises a static bond between podocytes and the GBM, and its expression is relatively stable. α3β1 integrin associates with the podocyte actin cytoskeleton through paxillin, talin, vinculin or α-actinin (Drenckhahn and Franke, 1988; Otey et al., 1993). Dystroglycan complex associates with the actin cytoskeleton through utrophin (Raats et al., 2000). Both the dystroglycan complex and α3β1 integrin attach to laminin and agrin of the GBM (Kerjaschki, 2001). Megalin belongs to the LDL-receptor family and serves as an endocytic receptor for lipoproteins (Kerjaschki et al., 1997).

2.3. Puromycin aminonucleoside nephrosis

The aminonucleoside of puromycin has been used to induce experimental proteinuric nephropathy. PAN has shown to be morphologically and functionally a useful experimental model for human minimal change nephropathy (Vernier et al., 1959). Minimal change nephropathy manifests usually at childhood and its typical features are proteinuria, hypoalbuminemia, hyperlipidemia, and occasionally haematuria (Glassock et al., 1991). Pathologic lesions include thickening of the capillary wall, subepithelial and intramembranous immune complex deposits together with disruption of podocyte foot process structure (Glassock et al., 1991). PAN may be induced with a single puromycin injection leading to proteinuria starting around day 3, peaking at day 10, and resolving by day 28 after injection (Ryan and Karnovsky, 1975). Injection of puromycin aminonucleoside leads to proteinuria in rats, which is characterized by detachment of the podocyte foot processes and GBM alterations (Caulfield et al., 1976). The number of foot processes is reduced, the foot processes are fused and the slit diaphragms are altered, even lost and replaced by occluding-type junctions (Caulfield et al., 1976; Kurihara et al., 1992b). The tubuli show dilation (Ryan and Karnovsky, 1975; Vernier et al., 1959) and finally the ruptured epithelium detaches from the GBM and allows direct contact of the GBM with the urinary space (Messina et al., 1987). Mice are generally resistant to the effects of puromycin, but proteinuria can be induced in mice with adriamycin possibly by a toxic effect mediated by the immune system (Amore et al., 1996; Chen et al., 1995). In some mouse strains repeated puromycin injections produce proteinuria (Pierce and Nakane, 1969). Both adriamycin and puromycin nephrosis mimic closely human minimal change nephropathy, but these toxins act most likely at different levels finally exerting their effects on protein synthesis. Adriamycin acts at the DNA level while puromycin acts on ribosomes (Whiteside et al., 1989).

Ahola et al. first showed that nephrin mRNA expression was reduced already at day 3 after PAN induction (Ahola et al., 1999), thus before proteinuria appeared. Kawachi et al. found that nephrin mRNA expression was reduced already two hours after puromycin injection by 51.2% when proteinuria was not yet present (Kawachi et al., 2000). Nephrin expression still decreased to a level of 20% of normal at day 10, as shown both in mRNA and protein levels (Luimula et al., 2000b). The nephrin staining pattern was altered from the basolateral area towards the more apical area in EM (Luimula et al., 2000b) and from a linear to a coarse granular appearance in immunofluorescence (Kawachi et al., 2000). Nephrin expression has also been found to be comparable to normal in areas where slits are well preserved, but lower in areas of foot process effacement (Lee et al., 2004). Luimula et al. found urinary nephrin of molecular size of 166 kDa in the most proteinuric urine samples (Luimula et al., 2000a). Podocin was down-regulated in PAN similar to nephrin (Luimula et al., 2002) although differing results also exist suggesting that pathogenic factors may cause disconnection of nephrin and podocin and result in an altered expression pattern (Kawachi et al., 2003). Saleem et al. reported that puromycin caused similar granular redistribution of both nephrin and actin in a podocyte cell line suggesting disruption of the actin-linked protein complex (Saleem et al., 2002). Expressional changes of other podocyte proteins in PAN are reviewed by Pavenstädt et al. (Pavenstadt et al., 2003).

Podocytes are particularly susceptible to toxic injury by oxidants. Overproduction of reactive oxygen species (ROS) through the xanthine oxidase pathway has been reported in PAN (Diamond et al., 1986). In vitro studies have shown that puromycin exerts an impact on rat glomerular epithelial cells by generation of active oxygen (Kawaguchi et al., 1992; Ricardo et al., 1994). Several studies have shown that antioxidants reduce proteinuria in PAN and inhibit foot process effacement (Diamond et al., 1986; Ricardo et al., 1994; Thakur et al., 1988). The major phenotypes in antioxidant-defective mouse overproducing ROS are podocyte injury and glomerulosclerosis (Binder et al., 1999). In PAN, podocyte depletion and glomerulosclerosis have a direct relationship (Kim et al., 2001). Probucol, a molecule that prevents lipid peroxidation, normalizes nephrin expression and prevents proteinuria in PAN (Luimula et al., 2000b). Administration of retinoid acid (vitamin A) to PAN rats ameliorated proteinuria and induced nephrin expression, but the exact pathway of this phenomenon is not yet known (Suzuki et al., 2003).

2.4. Apolipoprotein E

ApoE is a 34 kDa serum protein that mediates extracellular cholesterol transport and regulates multiple metabolic pathways. It is involved in the pathogenesis of atherosclerosis and Alzheimer's disease (Mahley and Huang, 1999). ApoE is a constituent of very low density lipoprotein synthesized by the liver, of intestinally synthesized chylomicrons, and of a subfraction of the high-density lipoproteins (Mahley, 1986). ApoE mediates high-affinity binding of ApoE-containing lipoprotein particles to the low density lipoprotein (LDL) receptor and is thus, among its other functions, responsible for the cellular uptake of these particles (Hui et al., 1981). The ApoE-knockout (ApoE-KO) mouse line was originally created using homologous recombination (Plump et al., 1992). These mice show high

cholesterol levels even when on low fat diet and have extensive atherosclerotic lesions at the age of ten weeks (Plump et al., 1992; Zhang et al., 1992). Elevated levels of very low and intermediate density lipoproteins are mainly responsible for the hypercholesterolemia in this model (Plump et al., 1992). Although the ApoE and total cholesterol levels in mice and men are different, the mouse ApoE knockout model has provided an invaluable insight into the roles of lipids and disease.

ApoE plays a role in the pathogenesis and progression of a variety of renal diseases, as well as in their atherosclerotic complications (Liberopoulos et al., 2004). Abnormal lipoprotein metabolism accelerates atherosclerosis and predisposes to the development of global glomerulosclerosis in patients with renal disease (Keane et al., 1988). For example increased Lipoprotein(a) level may contribute to accelerated atherosclerosis in ESRD patients (Milionis et al., 1999; Siamopoulos et al., 1995), whereas the ApoE polymorphism has been shown to influence the Lipoprotein(a) levels in nonuremic subjects (de Knijff et al., 1991). The polymorphisms of ApoE have been suggested to act as major determinants of plasma lipid levels of uremic patients (Liberopoulos et al., 2004). Certain mutations of the ApoE gene are associated with the unique and rare disorder, the lipoprotein glomerulopathy, which is characterized by nephrotic-range proteinuria without systemic manifestations (Saito et al., 2002). The histological features include presence of lipoprotein thrombi in capillary lumina of affected glomeruli, foam cells, vascular changes, and segmental sclerosis with periglomerular fibrosis in advanced stages of the disease (Saito et al., 1999). In normal glomeruli mesangial cells are the major expressors of ApoE and it has been speculated that ApoE may act as an autocrine regulator of mesangial and glomerular functions (Liberopoulos et al., 2004).

2.5. Type 1 diabetes

Finland has the world's highest incidence for type 1 diabetes mellitus (T1DM) being approximately 50 new annual cases per 100 000 children under the age of 15 years (Reunanen, 2004; Tuomilehto et al., 1999). The number of T1DM patients in Finland is now around 30 000 (Reunanen, 2004). The disease usually starts at an early age and is characterized by hyperglycemia caused by insulin deficiency leading to symptoms like weight loss, thirst and polyuria. Insulin-producing beta cells in the pancreas are slowly destroyed by an autoimmune mechanism launched by (polygenic) genetic and environmental factors. The autoimmune pre-diabetic process is characterized by T-cell infiltrations around the islets of Langerhans and finally inside the islets (Bottazzo et al., 1985; Gepts, 1965; Hanninen et al., 1992; Itoh et al., 1993). The patients carry several autoantibodies to beta cell autoantigens like glutamic acid decarboxylase (GAD65, GAD67), insulin and protein tyrosine phosphatase-related IA-2 molecule, and these antibodies are used to diagnose the pre-diabetic stage (Baekkeskov et al., 1990; Knip, 2002; Lan et al., 1996). HLA genotyping has been used also in evaluating subjects at risk for T1DM (Kupila et al., 2001). Although more than 90% of the patients with T1DM carry the predisposing HLA-DQ8 and/or –DQ2 alleles, only a minority of the genetically susceptible individuals progress to clinical disease (Kimpimaki et al., 2001b). There is evidence that environmental factors such as enterovirus infections (Hiltunen et al., 1997; Hyoty et

al., 1995), short-term breastfeeding (Kimpimaki et al., 2001a), and early induction of cow's milk-based infant formulas (Vaarala et al., 1999) may predispose genetically susceptible children to T1DM. Several intervention studies aimed at the prevention of T1DM are underway. The disease was untreatable until the discovery of insulin by Banting and Best in 1922 but although insulin replacement therapies are nowadays very good they are not completely able to mimic the physiological production of insulin.

2.6. Type 2 diabetes

The incidence of type 2 diabetes mellitus (T2DM) has increased during the last decades all over the world. The World Health Organization has estimated that there will be over 300 million diabetic patients in the world by the year 2025. In Finland there are now around 190 000 T2DM patients and the estimated number will be around 400 000 by the year 2030 (Reunanen, 2004). T2DM is a heterogeneous metabolic disorder characterized by defects both in insulin secretion and in insulin action (DeFronzo, 1988). T2DM can be present sub-clinically for many years (Harris et al., 1992) because symptoms of hyperglycemia manifest slowly and often the first symptoms are secondary, like infections. For many T2DM patients, insulin resistance is marked and forms part of the metabolic syndrome, which also includes central adiposity, hypertension, glucose intolerance, hypercoagulation tendency, microalbuminuria, and dyslipidemia (Alberti and Zimmet, 1998). Development of T2DM is, to some extent, predictable. Family history of diabetes and obesity are potent risk factors amplified by increasing age. In addition, both fasting hyperinsulinemia and fasting plasma glucose concentration independently indicate an enhanced risk of developing the disease (Haffner et al., 1990; Haffner et al., 1992). Insulin resistance and diabetes are not equivalent end points, and insulin resistance and beta cell dysfunction independently predict diabetes (Weyer et al., 2001). Several studies in different populations have identified anthropometrical and metabolic characteristics that increase the likelihood that a person with initially normal glucose tolerance will progress to diabetes over a specific period of time (Hanley et al., 2003; Harris et al., 1987; Zimmet and Whitehouse, 1978). Hanley et al. showed in a combined analysis of three prospective studies that the presence of one or more components of the metabolic syndrome, namely, hyperinsulinemia, dyslipidemia, hypertension, and glucose intolerance, predicted the emergence of diabetes over 8 years of follow-up (Hanley et al., 2003).

Concordance rates for T2DM are higher in monozygotic twins who share 100% of their genes, than in dizygotic twins who share less genes (Barnett et al., 1981; Newman et al., 1987). However, no consistent inheritance pattern has emerged, with some studies suggesting a major gene effect while others are more in keeping with polygenic inheritance. Nondiabetic first-degree relatives of T2DM patients have an almost threefold increased lifetime risk of diabetes in comparison to the background population. Insulin resistance is an early metabolic feature of nondiabetic first-degree relatives of T2DM patients (Eriksson et al., 1989) and also shows familial clustering in keeping with an underlying genetic predisposition (Lillioja et al., 1987). Maturity-onset diabetes of the young (MODY), a comparatively rare type of diabetes, is a monogenic disease and inherited as autosomal domi-

nant trait. MODY is characterized by beta cell dysfunction and young age at diagnosis, usually less than 25 years, leading to early-onset T2DM. There are at least six genes implicated in the pathogenesis of different forms of the disease (Frayling et al., 2001; Pearson et al., 2001).

2.7. Diabetic nephropathy

General pathologic complications caused by both T1DM and T2DM are usually divided into macrovascular and microvascular. The microvascular complications impair the function of small arteries partially by non-enzymatic glycosylation and the most common target organs are the kidneys, the peripheral nerves, and the eyes. Approximately one third of T1DM patients and one fifth of T2DM patients will eventually develop a diabetic kidney complication, diabetic nephropathy (DN). It is characterized by hypertension, persistent proteinuria, decline in renal function finally leading to renal failure and uremia. The first clinical sign of nephropathy is microalbuminuria caused by leakage of albumin to urine through the impaired glomerular filtration barrier. This albumin is detected by routine laboratory methods such as radioimmunoassay. Microalbuminuria is defined as a 24-h urinary albumin excretion rate (AER) of 30–300 mg in two of three consecutive 24-h urine collections and macroalbuminuria as AER >300 mg/24 h. From spot urine sample microalbuminuria may be determined by normalizing the excretion of albumin to creatinine. Microalbuminuric cut-off-points for albumin/creatinine ratios are 3.5 mg/mmol for women and 2.5 mg/mmol for men (Viberti et al., 1994).

The first histopathologic lesions of DN include enlarged glomeruli (hypertrophy, hyperplasia and glomerulomegaly), which is associated with increased glomerular filtration rate (GFR) (Mauer et al., 1984). At the microalbuminuric stage the glomerular basement membrane is thickened and there is mesangial matrix expansion, which may be accompanied by mild mesangial hypercellularity (Osterby et al., 1983). Overt glomerular matrix expansion (glomerulosclerosis) manifests as two basic patterns: diffuse glomerulosclerosis and nodular glomerulosclerosis. These two patterns often are present together in a biopsy specimen (Jennette, 2004). The nodular lesions of diabetic glomerulosclerosis were first described by Kimmelstiel and Wilson and are thus called Kimmelstiel-Wilson nodules (Kimmelstiel and Wilson, 1936). The nodules are often focal and segmental, although sometimes biopsies may show diffuse global nodularity. Glomerular hyalinosis is a common feature of diabetic glomerulosclerosis. Diabetic glomerulosclerosis is found in both type 1 and type 2 diabetes. In the latter it is somewhat more heterogeneous in appearance, in part because of concurrent changes caused by hypertension and aging (Bertani et al., 1996; Gambara et al., 1993). Atherosclerosis typically accompanies diabetic glomerulosclerosis. The earliest tubular change is thickening of the tubular basement membrane that is analogous to thickening of the GBM. With advancing disease, tubules become atrophic and the interstitium develops fibrosis and chronic inflammation. In EM the typical findings are thickening of the GBM, mesangial matrix expansion and hyalinosis (Jennette, 2004).

2.8. Factors affecting the pathogenesis of diabetic nephropathy

The landmark study that established the value of intensive blood glucose control to prevent the microvascular complications of T1DM was the Diabetes Control and Complications study (Anonymous, 1993). A few years later the UK Prospective Diabetes Study (UKPDS) fulfilled the same role for T2DM (Anonymous, 1998a, b). At the time of diagnosis of T1DM an increase in AER can be observed, which may become normal when glycaemic control improves (Mogensen, 1971). Also in non-diabetic subjects the prevalence of microalbuminuria increases with decreasing glucose tolerance (Collins et al., 1989). There are at least four main hypotheses that are proposed to explain how hyperglycemia causes diabetic complications: increased advanced glycation end-product (AGE) formation, increased polyol pathway flux, activation of PKC isoforms, and increased hexosamine pathway flux (Brownlee, 2001). It appears that intracellular hyperglycemia leads to formation of reactive, intracellular dicarbonyls, which react with amino groups of intracellular and extracellular proteins to form AGEs (Brownlee, 2001). The AGEs alter the structure and function of the intracellular proteins, and the extracellular matrix components modified by AGE precursors have altered function leading to altered cell to cell interaction. The plasma proteins modified by AGE precursors bind to AGE receptors on various cell types and induce receptor-mediated production of ROS leading to pathologic changes in gene expression and to vascular damage. Chronic hyperglycemia causes an increased flux of glucose via the polyol pathway and leads to accumulation of intracellular sorbitol. This may increase osmotic stress, induce activation of PKC or increase the intracellular oxidative stress in the cells, but the effects may be species, site, and tissue dependent (Brownlee, 2001). In vivo studies of inhibition of the polyol pathway have yielded inconsistent results. Activation of PKC isoforms by the lipid second messenger diacylglycerol (DAG) stimulates extracellular matrix production, expression of growth factors, and alters the function of vascular cells (Koya et al., 1997; Koya and King, 1998). Shunting of excess intracellular glucose into the hexosamine pathway might cause manifestation of diabetic complications possibly through transforming growth factor-β (TGF-β)-dependent increased mesangial matrix production (Kolm-Litty et al., 1998). Activation of the hexosamine pathway by hyperglycemia may result in alterations of gene expression and protein function. Recently, it was found that overproduction of superoxide by the mitochondrial electron-transport chain would activate all the four hyperglycemia-induced pathways and would thus be a common denominator for these four mechanisms (Du et al., 2000; Nishikawa et al., 2000).

Hypertension is a key player in the pathogenesis of DN, because intraglomerular pressure can increase protein filtration and finally cause mesangial expansion (Hostetter et al., 1982). Increased blood pressure is one of the key symptoms of DN, but it has also been thought that it may be secondary to the condition. Studies have shown that blood pressure lowering drugs, like ACE inhibitors, are able to postpone the development of DN in T1DM (Anonymous, 1996; Lewis et al., 1993; Mogensen et al., 1995). The same effect has been shown with angiotensin II type 1 receptor blockers on development of DN in T2DM patients (Brenner et al., 2001; Lewis

et al., 2001; Parving et al., 2001). Interestingly, in an experimental model AGE-RAGE-mediated ROS generation activated TGF-β-Smad signaling and subsequently induced mesangial cell hypertrophy and fibronectin synthesis by autocrine angiotensin II production in mesangial cells (Fukami et al., 2004).

Hyperlipidemia is one of the typical features of DN (Groop et al., 1996) but whether hyperlipidemia causes renal injury is not known. Abnormalities in lipid metabolism have been found already in microalbuminuric diabetic patients (Jensen et al., 1988; Tarnow et al., 1996). In a rat nephrectomy model of kidney injury a lipid-lowering agent clofibric acid reduced proteinuria (Kasiske et al., 1988) while cholesterol-lowering drug, lovastatin, did the same in diabetic rats (Inman et al., 1999). DN in T1DM has also been associated with genetic factors (Seaquist et al., 1989), smoking (Muhlhauser et al., 1986), high protein intake (Pedrini et al., 1996), and male gender (Seliger et al., 2001).

2.9. Nephrin in diabetic nephropathy

Streptozotocin (STZ) injection into rats causes rapid destruction of insulin-producing pancreatic beta cells leading to the phenotype of T1DM (Junod et al., 1967). Non-obese diabetic mice (NOD mice) spontaneously develop T1DM at the age of 3 to 6 months after T cell -mediated destruction of beta cells (Tisch et al., 1993). In these models it takes from four to eight weeks after the onset of diabetes to develop the first signs of nephropathy, enlargement of the glomeruli and albuminuria, if the blood glucose levels are not controlled well enough with insulin (Doi et al., 1990; O'Donnell et al., 1988). Aaltonen et al. showed that glomerular nephrin expression was increased by 50% in the STZ rats 4 weeks after induction of diabetes and a two-fold increase was present in 3 weeks old NOD mice even thought these mice did not demonstrate diabetes at that stage yet (Aaltonen et al., 2001). Whole-sized nephrin was found in the urine of the STZ-rats from 4 to 6 weeks after induction. Bonnet at al used STZ in spontaneously hypertensive rats and at 32 weeks the animals showed advanced DN together with clear reduction in both glomerular nephrin mRNA and protein levels (Bonnet et al., 2001). Very similar results have been observed in several other studies with an initial increase in nephrin expression after induction of diabetes followed by a later decrease in advanced DN (Forbes, 2002).

ACE inhibitors and angiotensin-receptor antagonists, which modulate the renin-angiotensin system (RAS), are known to reduce proteinuria (Lewis et al., 1993; Lewis et al., 2001). It has now been shown in several studies that these agents are able to normalize the decreased nephrin expression in experimental models of diabetes both at the mRNA and protein levels (Bonnet et al., 2001; Kelly et al., 2002). In a similar model the ACE inhibitor ramipril and angiotensin-receptor antagonist valsartan were able to normalize the structural alterations like podocyte foot process broadening and thickening of the GBM (Mifsud et al., 2001). RAS modifying agents are also able to modify the specific ZO-1 redistribution (Macconi et al., 2000). Podocytes express both type 1 and type 2 angiotensin II receptors and it has been shown that angiotensin II causes an increase in cyclic AMP and rearrangement of the actin cytoskeleton in podocytes, which is normalized by blocking simultaneously both receptors (Sharma et al., 1998). Stimulation of cultured podocytes with angiotensin II

or glycated albumin has been shown to cause a reduction in nephrin expression (Doublier et al., 2003). This was mediated through RAGE for glycated albumin and through cytoskeletal rearrangement for angiotensin II (Doublier et al., 2003). Controversial studies exist on treatment of diabetic rats with aminoguanidine, a blocker of AGE formation. One study showed no effect of aminoguanidine on nephrin expression in a STZ model, although it reduced proteinuria (Kelly et al., 2002), while another study showed normalization of nephrin expression in a similar model and even an additive effect with the ACE inhibitor, perindopril (Davis et al., 2004). Davis also showed that the vasopeptidase inhibitor, omapatrilat, was able to restore reduced nephrin expression in a similar model (Davis et al., 2003b). It is not surprising since vasopeptidase inhibitors simultaneously inhibit both ACE and neutral endopeptidase, a zinc dependent metallopeptidase. This leads to decreased levels of vasoconstrictor effector molecules such as angiotensin II as well as an increase in the levels of vasodilatory agents such as atrial natriuretic peptide and bradykinin (Fournie-Zaluski et al., 1994). It seems that the changes in nephrin expression are not only due to a reduction in blood pressure, since calcium channel blockers that reduced blood pressure equally effectively compared to angiotensin-receptor antagonist valsartan in a STZ model, had no effect on decreased nephrin expression (Davis et al., 2003a). Blanco et al. showed in a Zucker rat model that mimics T2DM that the ACE inhibitor quinapril increased nephrin expression while the calcium channel blocker diltiazem did not when compared to untreated diabetic animals (Blanco et al., 2005). Unfortunately this study did not compare the results to nondiabetic animals, so whether nephrin expression is altered per se in T2DM experimental model compared to nondiabetic animals remains unknown.

Langham et al. investigated renal biopsies from T2DM patients with proteinuria who had been randomized to receive the ACE inhibitor perindopril or placebo for two years. Nephrin mRNA was reduced in diabetic patients compared to healthy controls by 62% while the levels of perindopril treated patients were similar to the levels of the controls (Langham et al., 2002). Doublier et al. found a reduction in nephrin protein levels both in T1DM and T2DM patients with nephrotic syndrome (Doublier et al., 2003). They found a profound reduction in nephrin staining already in patients with microalbuminuria and that the staining pattern was changed to granular from the normal linear. Koop et al. showed that nephrin protein expression was reduced in biopsies of DN patients, while podocin and podocalyxin staining was comparable to that of normal controls (Koop et al., 2003). In this study they found inverse correlation between nephrin protein levels and mean width of the podocyte foot processes but no correlation between nephrin and serum creatinine. Toyoda et al. showed an inverse correlation between glomerular nephrin mRNA levels and proteinuria in T2DM patients with DN (Toyoda et al., 2004). Benigni et al. reported that in diabetic nephropathy of T2DM extracellular nephrin staining was reduced while staining with nephrin antibody against the intracellular domain was normal suggesting a possible diabetes-associated nephrin splicing (Benigni et al., 2004). None of the human studies has assessed nephrin expression in normoalbuminuric diabetic patients.

3. AIMS OF THE PRESENT STUDY

Discovery of the pathogenic process of the CNF has provided us with a deeper understanding of the molecular structure of the glomerular filtration diaphragm and knowledge that nephrin is a key molecule in the filtration function. The aims of this thesis are the following:

1. To study the role of nephrin and lipid peroxidation in glomerular damage in the novel hypercholesterolemic PAN mouse model (I)

2. To study the presence of urinary proteins, detected with nephrin antisera, in the urine of type 1 diabetic patients with or without nephropathy (II)

3. To identify nephrin among the proteins found in the urine of type 1 diabetic patients (III)

4. To study whether the 75 kDa urinary nephrin can be used as a marker for progression of diabetic nephropathy in type 1 diabetes (III)

5. To study whether offspring of type 2 diabetic patients exhibit urinary proteins detectable with nephrin antiserum and whether presence of these proteins associate with mediators of glucose metabolism, especially with insulin resistance (IV)

4. MATERIALS AND METHODS

4.1. Tissues

Normal kidney tissue was obtained from Department of Surgery (University of Helsinki, Finland) from cadaver kidneys taken for transplantation but not grafted because of vascular anatomic abnormalities in accordance with the principles of the Declaration of Helsinki. Kidney cortex was stored at –70°C, and the tissue was used as such or further prepared for glomerular isolation by graded sieving method (Holthofer et al., 1994; Striker and Striker, 1985). Collected glomeruli were aliquoted and stored in –70°C for lysate preparation (Study IV, Research design and methods).

4.2. Animals

The ApoE knockout mice were housed in controlled humidity and temperature in the animal facility of University of Tampere, Finland. The procedures were approved by the ethics committee of the University of Tampere. The mice were randomly assigned to two main dietary groups: apoE group-1 fed with normal mouse chow diet, and apoE group-2 fed with a high fat diet. The mice were further divided into four treatment subgroups: puromycin, puromycin + probucol, probucol and control as shown in Table 4.1 (See details in Study I, Methods). The PAN was induced by a single 15 mg/100 g intraperitoneal injection of puromycin (Sigma Chemicals Co, St Louis, MO, USA) and the control group received an equal volume of 0.9% saline. Probucol (Sigma Chemicals) was given in the diet (2% wt/wt) and consumption was recorded daily.

Table 4.1. Experimental design of Study I

		Treatment			
High fat diet (ApoE group-2)	N	-10 days	0 days	3 days	8 days
PAN	3+3		U, PAN	U, B, K	U, B, K
				† (n=3)	† (n=3)
PAN+Pro	3+3	Pro	U, PAN	U, B, K	U, B, K
				† (n=3)	† (n=3)
Pro	3+3	Pro	U, saline	U, B, K	U, B, K
				† (n=3)	† (n=3)
Control	3+3		U, saline	U, B, K	U, B, K
				† (n=3)	† (n=3)

		Treatment			
Normal mouse diet (ApoE group-1)	N	-10 days	0 days	3 days	8 days
PAN	2		U, PAN		U, B, K †
PAN+Pro	2	Pro	U, PAN		U, B, K †
Pro	2	Pro	U, saline		U, B, K †
Control	2		U, saline		U, B, K †

PAN, aminonucleoside of puromycin; Pro, probucol; †, sacrifice; U, urine sample; B, blood sample; K, kidney sample

4.3. Measurement of nephrin mRNA expression

Cortical kidney RNA was isolated from the frozen mouse tissues using the single-step acid guanidium thiocyanate-phenol-chloroform procedure with Trizol® reagent (Life Technologies, Gibco BRL, Paisley, UK) according to manufacturer's instructions. For removal of genomic DNA, the total RNA was incubated with Dnase I (Promega, Madison, WI, USA) together with Rnase inhibitor (Promega) for 30 min at 37°C. Using oligo dT15 primer (Roche Diagnostics GmbH, Mannheim, Germany) and Moloney-Murine Leukemia Virus reverse transcriptase (Promega) RNA was transcribed into cDNA followed by quantification of nephrin expression by Taqman® Real-Time PCR ABI Prism® 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems, Norwalk, CT, USA). In this method, a probe (5'-ccctctctaaatgcacggccacca-3') with a 5'-reporter dye FAM® (6-carboxy-fluorescein) and a 3'-quencher dye TAMRA (6-carboxy-tetramethylrhodamine), and a primer pair 5'-atctccaagaccccaggtacaca-3' (forward) and 5'-agggtcagggcgctgat-3' (reverse) were used for amplification of mouse nephrin cDNA. Taqman Universal Master Mix was used in all PCR reactions. Finally, nephrin mRNA level of each mouse was compared to its respective GAPDH (glyceraldehydes-3-phosphate dehydrogenase; housekeeping gene) mRNA level.

4.4. Type 1 diabetic patients and controls

The type 1 diabetic patients (n=159) of cross-sectional cohort of Study II and Study III (Cohort I) were from the FinnDiane study (Department of Medicine, Division of Nephrology, Helsinki University Central Hospital and Folkhälsan Research Centre, Biomedicum Helsinki, Finland). FinnDiane is an ongoing, multicenter, nationwide study that aims at characterizing 25% of the Finnish type 1 diabetic population. The type 1 diabetic patients were divided into four groups according to AER-measurements: Normoalbuminuric (Normo in Study II, Normo-I in Study III, n=40), microalbuminuric (Micro, Micro-I, n=41), macroalbuminuric (Macro, Macro-I, n=39) and new microalbuminuric (newMicro, newMicro-I, n=39) groups. The newMicro consisted of patients previously normoalbuminuric, but the urine sample analyzed in the study was the first showing microalbuminuric range AER. The Macro patients had recent onset (<2 years) of diabetic nephropathy. Healthy nondiabetic laboratory personnel (n=29) were used as control subjects. For detailed clinical characteristics of the diabetic study subjects and healthy controls see Study II; Table 1, and Research design and methods.

The Study III follow-up patients (Cohort II) were recruited from the Outpatient Clinic of the Department of Ophthalmology, Helsinki University Central Hospital, during years 1980-1981. The patients were re-examined 7.8 years later. Research design and methods are described in detail in Study III. Every patient gave a written informed consent, and the studies were approved by the local ethics committees.

4.5. Offspring of type 2 diabetic patients and controls

For Study IV 128 healthy offspring of type 2 diabetic patients and 9 control subjects were studied. The diabetic patients (probands) were randomly selected among type 2 diabetic patients living in the region of Kuopio University Hospital. Spouses of the probands had to have a normal oral glucose tolerance test (OGTT).

One to three offspring from each family were included in metabolic studies (details in Study IV; Research design and methods) of which OGTT, intravenous glucose tolerance test (IVGTT), and euglycemic hyperinsulinemic clamp (clamp) techniques are explained briefly below. All study subjects gave written informed consent and the study was approved by the Ethics Committee of the University of Kuopio.

Table 4.2. Summary of subjects and urine samples in Studies II, III and IV

	N	Urine sample	Classification	Used in
Type 1 diabetic patients, Cohort I	159	24-h urine	AER	II, III
Type 1 diabetic patients, 7.8-years follow up, Cohort II	73	24-h urine	AER	III
Offspring of type 2 diabetic patients	128	Timed overnight urine	AER	IV
Healthy control subjects, uncharacterized	29	Morning urine	Alb/Crea	II
Healthy control subjects, characterized by metabolic studies	9	Timed overnight urine	AER	IV

4.6. Oral glucose tolerance test (OGTT), intravenous glucose tolerance test (IVGTT) and euglycemic hyperinsulinemic clamp (clamp)

Glucose tolerance tests are used to determine the ability of an individual to maintain homeostasis of blood glucose. It includes measuring blood glucose levels in the fasting state and at prescribed intervals before and after oral glucose intake (OGTT) or intravenous infusion (intravenous glucose tolerance test, IVGTT). OGTT is widely used for detecting impaired glucose tolerance, *i.e.* a state with higher than normal blood glucose, but not high enough to establish a diagnosis of diabetes. After a 12-hours fast a 75 g glucose dose is given orally and samples for blood glucose and plasma insulin measurements are drawn at –10, 0, 30, 60 and 120 min. For determining the first-phase insulin secretion capacity after a 12-hours fast an IVGTT is performed. In this method a bolus of glucose (300 mg/kg as a 50% solution) is given within 30 sec into the antecubital vein. Blood glucose and plasma insulin samples (arterialized venous blood) are drawn at -5, 0, 2, 4, 6, 8, 10, 20, 30, 40, 50 and 60 min.

Insulin sensitivity can be evaluated with the euglycemic hyperinsulinemic clamp technique (clamp) using insulin infusion rate of 240 pmol/min/m^2 body surface area. Blood glucose for the next 120 min is maintained at 5.0 mmol/l by infusing 20% glucose at varying rates according to blood glucose measurements performed at 5-min intervals. Indirect calorimetry before the clamp and during the last 20 min of the clamp can be coupled to the technique using a computerized flow-through canopy gas analyzer system (DELTATRAC®, TM Datex, Helsinki, Finland) (Vauhkonen et al., 1998). Mean values of the data during the last 20 min of the clamp are used to calculate the M-value (whole body glucose uptake; glucose infusion μmol/kg lean body mass/min), glucose oxidation and lipid oxidation. The rates of nonoxidative glucose disposal during the clamp may be estimated by subtracting the rates of glucose oxidation from the glucose infusion rate.

4.7. Antibodies used

Table 4.3.

Primary antibodies

Name	Antigen	Source	Dilution or concentration	Used in
MDA (616)	Mouse malondialdehyde	Rabbit polyclonal, Dr. T. Montine (Montine et al., 1996)	IF 1:50	I
4-HNE (614)	Mouse 4-hydroxynonenal	Rabbit polyclonal, Dr. T. Montine (Montine et al., 1996)	IF1:50	I
Anti-nephrin #6878	Mouse nephrin	Rabbit polyclonal, Dr. L. Holzman (Holzman et al., 1999)	IF 1:100	I
Aff338	Human nephrin, recombinant protein alpha-435: aa1031-1055 and 1096-1215	Rabbit polyclonal, rabbit 338	WB 1:5 IF 1:1	II, IV
Aff380	Human nephrin, recombinant protein alpha-435: aa1031-1055 and 1096-1215	Rabbit polyclonal, rabbit 380	WB 1:5 IF 1:1	II
#1188	Human nephrin, recombinant protein alpha-435: aa1031-1055 and 1096-1215	Rabbit polyclonal, protein A –purified, rabbit 338	15 ug/ml	III
#1135	Human nephrin, recombinant protein alpha-435: aa1031-1055 and 1096-1215	Rabbit polyclonal, protein A –purified, rabbit 380	15 ug/ml	III
Glucagon	Human glucagon	Rabbit polyclonal, Zymed	IF 1:50	II

Secondary antibodies

Name	Antigen	Source	Dilution	Used in
FITC-anti Rb IgG	Rabbit IgG	Rat polyclonal, FITC-conjugated, Dako	IF 1:100	I
TRITC-anti Rb IgG	Rabbit IgG	Goat polyclonal, TRITC-conjugated, Jackson	IF 1:200	II
HRP-anti Rb IgG	Rabbit IgG	Goat polyclonal, HRP-conjugated, Jackson	WB 1:40 000	II, III, IV

4.8. Immunofluorescence microscopy

Frozen (-70°C) kidney cortexes were embedded in Tissue-Tek® mounting medium (Sakura) and 4-5 µm cryosections were cut. The sections were either fixed with ice-cold (-20°C) acetone for 5 min or air-dried for 30 min before fixing with acetone. After washing with PBS the sections were incubated with primary antibody in 1% goat or 5% rat serum in PBS overnight at 6°C. The sections were washed with PBS followed by incubations with secondary antibody at room temperature for 30 min. The slides were covered with mounting medium (Shandon, Pittsburgh, PA, USA) and examined using an Olympus BX50 microscope (Olympus Optical, Tokyo, Japan) equipped with a CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Openlab 2.2.3 (Improvision, Coventry, U.K.) and Adobe Photoshop (Adobe Systems, San Jose, CA, USA) software was used for image documentation. For Study I a dilution series of the primary antibody was used to assess the level of nephrin protein expression. See details of the incubation and microscopy conditions in Study I (Methods) and Study II (Research design and methods).

4.9. Determination of urinary proteins

The mouse urinary albumin concentration was measured by nephelometry (Luimula et al., 2000b). The human urine samples used and the methods for classification of albumin excretion rate are listed in Table 4.2. Urinary albumin concentration was measured using radioimmunoassay in Study II. Alternatively, albumin was determined by immunoturbidometry (Study III) or by kinetic nephelometry (Study IV).

Total urinary protein concentration was measured with the Lowry method using RC DC Protein Assay kit (Bio-Rad Laboratories, Helcules, CA, USA) according to the manufacturer's instructions. The concentration of non-albumin urinary proteins was calculated as the difference between the concentration of total protein minus the concentration of albumin. A larger urine volume corresponding to 30 µg of non-albumin proteins was used in Studies II and III. Because the albumin/total protein ratio of 42 samples in Study IV correlated with the ratios of Normo in Study II, we were able to calculate the total urinary protein concentration for the rest of Study IV samples using the abovementioned formula and albumin concentration.

4.10. Western blotting

Sample volumes corresponding to 30 µg of total protein or 30 µg of non-albumin proteins (microalbuminuric and macroalbuminuric groups in Studies II and III) were precipitated with 10% (wt/vol) trichloroacetic acid in PBS on ice for 30 min. The samples were centrifuged for 10 min at 13,100g at 4°C and the precipitate was washed twice with ice-cold acetone. The samples were air-dried and dissolved in Laemmli buffer (62.5 mmol/l Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue) followed by heating at 95°C for 5 min. The samples were analyzed in 10% polyacrylamide gels with the Protean Mini-gel electrophoresis system using Ready Gels (Bio-Rad Laboratories). A nephrinuric urine sample from a type 1 diabetic patient was used as a positive control in every gel. After the run the proteins were transferred onto nitrocellulose filters (Amersham Biosciences, Buckinghamshire, UK)

followed by blocking for two hours at room temperature (RT) with 3% non-fat dried milk (Valio, Helsinki, Finland) in PBS. The filters were incubated with the primary antibody (listed in Table 4.3) in PBS containing 1% non-fat dried milk and 0.02% sodium azide at RT (for 1 hour in Study II and III, and 1.5 hours in Study IV), and then washed several times in PBS containing 0.2% Tween 20. Then the filters were incubated with horseradish peroxidase -labeled secondary antibody for one hour at RT, and washed as above. The bound antibody was detected with Super Signal ECL substrate (Pierce, Rockford, IL, USA). Presence of any protein band visible with both antibodies in Western blots was regarded as positive for nephrinuria in Study II.

4.11. Absorption of antisera

The E. coli strain TOP10 (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for production of the alpha-435 recombinant fusion protein immunogen (Figure 2.4). A lysate of the nontransfected strain was produced as described in Study III. The primary polyclonal nephrin antiserum (5 ml of 15 µg/ml of IgG in PBS containing 1% non-fat dried milk and 0.02% sodium azide) was incubated with alpha-435 antigen (100 µg and 600 µg) for four hours at RT to absorb the nephrin specific antibodies in the antiserum or alternatively with TOP10 lysate (100 µg and 600 µg) to absorb the E. coli specific antibodies. The absorbed antisera were then used as primary antibodies for Western blottings of the test samples (a positive urine sample from a nephrinuric type 1 diabetic patient and glomerular lysate) in Study III.

4.12. Statistical analyses

Data were analyzed with BMDP statistical package (BMDP Statistical Software, Los Angeles, CA, USA) in Study II, and with the SPSS for Windows program (SPSS Inc., Chicago, IL, USA) in Studies III and IV. Differences between the groups were tested using analysis of variance (ANOVA). A P-value <0.05 was considered statistically significant. Regression analysis was performed to evaluate the association of different variables with whole body glucose uptake (M/I) in Study IV.

4.13. Miscellaneous

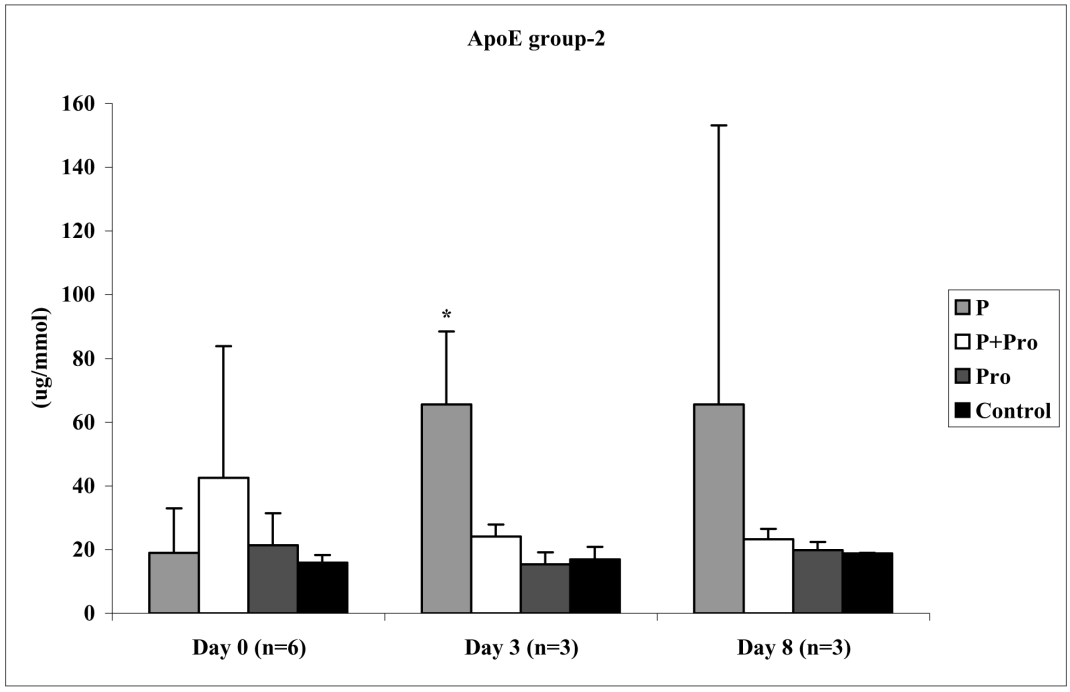
Serum cholesterol and triglyceride concentrations were measured enzymatically in Study I (Friedewald et al., 1972). Routine clinical chemistry was performed in the central laboratories of Kuopio and Helsinki University Central Hospitals in Studies II, III and IV.

5. RESULTS

5.1. Hypercholesterolemia is a prerequisite for glomerular damage in the proteinuric PAN mouse model (I)

ApoE mice on a high fat diet (apoE group-2) had significantly higher serum cholesterol values compared to the values of mice on a normal diet (Study I, Table 1). Interestingly, puromycin treated animals on high fat diet group had 34% lower cholesterol than control animals on same diet. Probucol lowered the cholesterol level alone and together with puromycin by 70%. Puromycin was able to induce proteinuria in the apoE mice on a high fat diet, but not in apoE mice on a normal diet. The difference was most remarkable at day 3 as shown in Figure 5.1 (see also Study I, Figure 1). Probucol treatment prior to puromycin injection was able to prevent proteinuria.

Figure 5.1 Urinary albumin-to-creatinine ratios of ApoE mice on high fat diet.



*p=0.05 P=Puromycin, Pro=Probucol

5.2. Nephrin expression and lipid peroxidation in hypercholesterolemic PAN mouse model (I)

Nephrin mRNA expression decreased by 30% and 50% at days 3 and 8 after puromycin injection, respectively, but the changes were not statistically significant (Study I, Figure 3). Probucol was able to increase nephrin expression both alone and when used with puromycin, although not statistically significantly. At the protein level puromycin reduced nephrin expression in the glomeruli of apoE group-2 mice, but not when probucol was used prior to puromycin injection (Study I, Figure 4 and Table 2).

The immunostaining for MDA and 4-HNE was increased at day 3 in puromycin-treated animals of apoE group-2 (Study I, Figure 2). In mice treated with probucol alone or probucol with puromycin, the staining was comparable to that of controls. The staining of MDA and 4-HNE appeared to localize to the mesangial part of the glomeruli, although granular staining was also noticed next to the urinary space.

5.3. Urinary proteins detected by nephrin antisera in type 1 diabetic patients with or without nephropathy (II)

In Study II the criterion for nephrinuria was whether there was any protein band in urine that was visible with both of two different nephrin polyclonal antisera against the same immunogen alpha-435. Using this criterium, 30% of Normo patients were nephrinuric, while 28% of newMicro and Macro patients. Of Micro patients 17% were nephrinuric, while all healthy control subjects were negative (Study II, Figure 1). Of all female diabetic patients 35% but only 19% of all male patients were nephrinuric (P=0.02). Nephrinuric and non-nephrinuric patients did not differ from each other in respect to the variables listed in Study II; Table 1. The nephrin antisera revealed protein bands in urine most commonly of sizes 32, 40, 60, and 75 kDa (Study II, Figure 3). The antisera Aff338 and Aff380 also recognized full-length nephrin (185kDa) in human glomerular lysate (Study II, Figure 3) and produced typical immunostaining of nephrin in human glomeruli (Study II, Figure 4).

5.4. Specificity of the urinary proteins found in type 1 diabetic patients (III)

To investigate which of the urinary proteins found in the urine of T1DM patient would be most specific for nephrin a specificity assay was conducted with absorbed antibodies. In the 75 kDa area a distinct and closely packed doublet of protein bands was detected and this doublet was called 75 kDa nephrin. 32 and 40 kDa bands weakened when the polyclonal nephrin antiserum was absorbed with TOP10 *E. coli* lysate suggesting that these band were not nephrin-derived (Study III, Figure 1). However, both the 75 kDa urinary nephrin and 185 kDa glomerular nephrin remained positive with this absorbed antibody, and disappeared when the antibody was absorbed with the immunogen, alpha-435 (Study III, Figure 1).

5.5. The occurrence of 75 kDa nephrin is highest in normoalbuminuric type 1 diabetic patients and diminishes when diabetic nephropathy progresses (III)

According to the results of the specificity assay using absorbed antibodies the 75 kDa protein was nephrin-derived. We reanalyzed the type 1 diabetic patients of Study II, called the Cohort I in Study III, for the presence of this particular protein. The analysis revealed that of Normo-I, newMicro-I, Micro-I, and Macro-I 22.5%, 23.1%, 9.8%, and 2.6% (p=0.022), respectively, were positive (Study III, Figure 2). There were no significant differences in clinical variables such as duration of diabetes, BMI, systolic blood pressure, diastolic blood pressure, serum lipid levels or glycated hemoglobin levels between subjects positive or negative for 75 kDa nephrin. Of all diabetic female patients in Cohort I, 21.7% were nephrinuric compared with only 8.9% of the male patients (p=0.022). In Cohort II 75 kDa nephrin occurred in 45.4%, 26.4%, and 0% (p=0.001) in Normo-II, Micro-II and Macro-II groups, respectively (Study III, Figure 3). Patients positive or negative for 75 kDa nephrin did not differ significantly from each other with respect to age, sex, duration of diabetes, BMI, serum lipid levels, serum creatinine, creatinine clearance or glycated hemoglobin. In total, four patients in the Normo-II group and six patients in the Micro-II group progressed. Of these progressors only two out of ten were positive for 75 kDa nephrin at baseline while this was the case in 15 out of 36 nonprogressors (p=0.282; Study III, Figure 4).

5.6. Offspring of type 2 diabetic patients exhibit urinary proteins detectable with a nephrin antiserum (IV)

Of all offspring 26.6% showed a 100 kDa urinary protein in the Western blots that stained with the Aff338 antiserum (Study IV, Figure 1), while all control subjects were negative. The subjects were divided into strongly positive (12.5%), weakly positive (14.1%) and negative groups and compared for the clinical characteristics. The offspring had lower HDL cholesterol than the healthy controls (Study IV, Table 1). The strongly positive offspring showed a trend towards lower HDL cholesterol, higher BMI, higher percentage of smokers, and higher fasting and 120 minutes insulin levels in OGTT, although these differences were not statistically significant. Interestingly, the nephrin antiserum occasionally detected a 100 kDa protein in glomerular lysate. Altogether 79% of offspring and 78% of control subjects showed a urinary protein band of size 185-200 kDa (Study IV, Figure 1). Subjects positive or negative for this band did not differ with respect to clinical and biochemical characteristics.

5.7. The 100 kDa urinary protein is associated with insulin resistance in the offspring of type 2 diabetic patients (IV)

During the first 10 min of the intravenous glucose tolerance test the strongly positive group had a higher insulin AUC value than the negative group (3700 ± 706 vs. 2306 ± 159 pmol/lxmin, P=0.007), but the insulin AUC of the weakly positive group (2456 ± 345 pmol/lxmin) did not differ significantly from those of the other groups (Study IV, Figure 2). During the last 20 min of the euglycemic hyperinsulinemic clamp the in-

sulin levels of the strongly positive offspring were higher compared to the levels of negative offspring (459.6 ± 29.9 pmol/l vs. 389.4 ± 8.3 pmol/l, P=0.003) and tended to be higher than the levels of weakly positive offspring (427.8 ± 17.2 pmol/l). The strongly positive offspring had lower insulin sensitivity than the negative offspring (11.3 ± 1.2 vs. 15.8 ± 0.6 µmol/kg/min/pmol/l, P=0.007) as expressed by whole body glucose uptake (M/I) normalized to plasma insulin concentrations during the last 20 minutes of the euglycemic hyperinsulinemic clamp (Study IV, Figure 3). After adjustment for the insulin AUC values during the first 10 minutes of the IVGTT (ANCOVA) the difference in M/I between the groups disappeared suggesting that the subjects positive for the 100 kDa urinary protein were capable of compensating their insulin resistance by increased insulin secretion. Nonoxidative glucose disposal was lower in the strongly positive group compared to the negative group (6.4 ± 0.9 vs. 10 ± 0.5 µmol/kg/min/pmol/l, P=0.007) but did not differ significantly from that of the weakly positive group (9 ± 1.3 µmol/kg/min/pmol/l). Multiple regression analysis showed that the presence of 100 kDa urinary protein was associated with the rates of M/I and non-oxidative glucose disposal independently of several factors associated with insulin resistance (Study IV, Table 2). Subjects positive or negative for the 185-200 kDa urinary protein did not show any difference in first phase insulin secretion during IVGTT or in insulin sensitivity during the clamp.

6. DISCUSSION

6.1. Proteinuria, lipid peroxidation, and nephrin expression in the PAN model of hypercholesterolemic ApoE mice

The rat proteinuric PAN model has been widely used to mimic human minimal change disease, while mice are rather resistant to puromycin and have generally not shown proteinuria. In Study I only ApoE mice on a high fat diet with overt hypercholesterolemia were prone to puromycin-induced proteinuria. Probucol reduced both cholesterolemia and proteinuria suggesting that hypercholesterolemia, actually, may act as a risk factor for proteinuria, rather than being a consequence of kidney failure. In humans hypercholesterolemia is a frequent finding in glomerular diseases (Kaysen et al., 1986; Keane et al., 1988), like in DN (Groop et al., 1996) although the mechanisms for this remain obscure. Microalbuminuric diabetic patients already show lipid abnormalities (Tarnow et al., 1996) and this is also the case in patients with the metabolic syndrome (Eckel et al., 2005). It is also known that with age ApoE-KO mice develop mild progressive renal injury with spontaneous glomerular lesions with foam cells and widening of the mesangial area resembling changes in human type III hyperlipoproteinemia (Wen et al., 2002).

Lipid peroxidation is directly associated with glomerular damage as shown in the Heymann nephritis model of membranous glomerulonephritis (Neale et al., 1994; Neale et al., 1993) and in the PAN model (Gwinner et al., 1997). Puromycin may mediate its functions through mitochondrial damage (Goldenberg et al., 2005; Solin et al., 2000) or through production of ROS via the xanthine oxidase pathway (Diamond et al., 1986). Adenosine deaminase may also be involved in this pathway by regulating production of ROS, since inhibition of adenosine deaminase prevents proteinuria in rat PAN (Nosaka et al., 1997). However, the mechanisms by which ROS and particularly lipid peroxidation may cause renal disease remain to be defined. When ROS react with lipids, various adducts are formed, of which MDA and 4-HNE are some of the most abundant ones. MDA reacts with DNA and is mutagenic (Marnett, 2002), while 4-HNE stimulates neutrophil chemotaxis and activates enzymes like phospholipase C leading to altered cellular functions (Dianzani, 2003). Probucol is a lipid antioxidant and it has also previously been shown to lower lipoprotein levels and proteinuria in PAN (Hirano et al., 1991). In Study I we found that puromycin-treated hypercholesterolemic mice showed increased levels of MDA and 4-HNE in the glomeruli, especially in the mesangial area. When given before puromycin, probucol prevented these changes. Taken together, these results suggest that high cholesterol, formation of ROS, and lipid peroxidation contribute to the development of proteinuria.

In our study glomerular nephrin mRNA expression was diminished in hypercholesterolemic mice by 30% and 50% at days 3 and 8 after puromycin injection, respectively. The protein expression of nephrin was also diminished in proteinuric mice. Probucol reduced proteinuria and nephrin expression to the level of the control mice. These results are consistent with previous studies using the PAN model, in which nephrin staining also was reported to change from a linear

to more granular pattern (Kawachi et al., 2000; Luimula et al., 2000b). Using immuno-EM Luimula et al. observed that nephrin shifted from slits to an apical position on podocytes. Similar results were observed in another study, but nephrin expression was altered only in areas with foot process effacement, while in preserved areas the localization of nephrin was normal (Lee et al., 2004). Kawachi et al. reported that podocin expression is also diminished in PAN but it did not shift to an apical localization but partly remained in the slit area in newly formed tight junctions. Furthermore, they suggested that podocin was excreted into urine (Kawachi et al., 2003). Podocin and podocalyxin have been found being excreted in urinary exosomes (Knepper, 2004). It has also been shown that podocyte loss and glomerulosclerosis are associated in the PAN model (Kim et al., 2001). At the same time podocytes appear in urine as evidenced by detection of nephrin mRNA (Kim et al., 2001). Interestingly, foot process effacement is preceded by induction of α-actinin and α3β1 integrin mRNA in PAN, suggesting that morphological changes *i.e.*, alterations in podocyte proteins and disturbed interaction of proteins with GBM and with the actin cytoskeleton may lead to proteinuria (Luimula et al., 2002; Smoyer et al., 1997). Puromycin caused a change to granular distribution of nephrin, podocin, and actin fibers in a human podocyte cell line (Saleem et al., 2002). This effect was similar to that of cytochalasin, an agent that disrupts actin stress fibers (Saleem et al., 2002). Changes in nephrin expression in PAN may thus be secondary to alterations in the actin cytoskeleton leading to disturbed podocyte morphology. B7-1 (CD80) is not expressed in the normal podocyte, but its mRNA is up-regulated in puromycin-treated podocytes. This was suggested to contribute to the pathogenesis of proteinuria by disrupting the slit diaphragm protein complex and by reorganizing the actin cytoskeleton (Reiser et al., 2004).

Megalin is a protein located between the podocyte and the GBM. It belongs to the LDL receptor family and could mediate some of the effects of hypercholesterolemia by acting as an endocytic receptor for lipoproteins (Kerjaschki et al., 1997). Some of the effects of hypercholesterolemia could also be mediated through the RAS, since hypercholesterolemia increases angiotensin II type 1 receptors in vascular smooth muscle cells leading to an increase in ROS (Griendling et al., 1994; Nickenig et al., 1997). Blocking the RAS with ACE inhibitors or angiotensin-receptor antagonists reduces proteinuria (Lewis et al., 1993; Lewis et al., 2001; Parving et al., 2001). These agents also normalized changes in nephrin expression in a proteinuric model of Heymann nephritis (Benigni et al., 2001). Interestingly, ACE inhibitors have beneficial effects even in the treatment of some CNF patients (Guez et al., 1998; Pomeranz et al., 1995). Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, *i.e.* statins, are effective lipid lowering drugs and statin treatment protects kidneys from ischemia-reperfusion injury in a uninephrectomized rat model (Gueler et al., 2002). The effects are possibly mediated through the nuclear factor-κB pathway (Gueler et al., 2002), which also mediates angiotensin II signaling (Ruiz-Ortega et al., 2000). Taken together, the results suggest a direct but as yet unknown relationship between serum lipids and proteinuria. Interestingly, induction of diabetes with streptozotocin in the ApoE-KO mice resulted in accelerated renal injury (Lassila

et al., 2004). The increase in albuminuria was attenuated by treatment with an inhibitor of AGE formation and with a cross-link breaker that cleaves the preformed AGE (Lassila et al., 2004). The study suggested that AGE is not only derived from glucose-dependent pathways but that lipids may also contribute to the accumulation of AGE. Attenuation of renal injury by AGE inhibitors was associated with reduced expression of profibrotic and proinflammatory substances like TGF-β1 and collagens (Lassila et al., 2004).

6.2. Podocyturia, nephrin, and nephrinuria in type 1 diabetes

The number of podocytes appears to be reduced in both T1DM (Steffes et al., 2001) and T2DM (Dalla Vestra et al., 2003; Pagtalunan et al., 1997). A study of T2DM patients showed that together with GBM thickening the podocyte foot processes were broadened, the podocyte number was reduced and the filtration surface area covered by remaining podocytes was increased (Pagtalunan et al., 1997). It has been shown that approximately one in five podocytes is reduced in T1DM of short duration indicating an increased risk for functional abnormalities as diabetes progresses (Steffes et al., 2001). In another study on T1DM patients no changes in podocyte number were detected compared to healthy controls, but during follow-up there was an association between podocyte loss and increased AER (White et al., 2002). In T2DM patients a reduced number of podocytes predicts rapid progression of renal disease (Meyer et al., 1999) and an increase in AER (Dalla Vestra et al., 2003). It is believed that podocytes are incapable of replication and have a limited potential for repair (Kriz et al., 1998). Interestingly, viable detached podocytes have been found in urine of proteinuric rats (Petermann et al., 2003) and patients with glomerular disease (Vogelmann et al., 2003). In the study by Vogelmann et al, the patients had focal segmental glomerulosclerosis and Lupus nephritis, and over 80% of these patients had podocalyxin-positive cells, regarded as podocytes, in the urine (Vogelmann et al., 2003). Interestingly, 44% of healthy controls also had podocyturia. Podocalyxin is also expressed in human peripheral blood leucocytes at the mRNA, but not on the protein level (Kerosuo et al., 2004). Thus there is a possibility that podocalyxin-expressing cells in urine are not podocytes. Indeed in Vogelmann's study, only 30-40% of the samples positive for podocalyxin were positive for other podocyte markers, such as synaptopodin, GLEPP1, or podocin. Part of the cells were apoptotic and podocyturia tended to be highest in patients with the lowest levels of albuminuria (Vogelmann et al., 2003). Podocalyxin was also used as a marker for urinary podocytes in a study of T2DM patients: 53% and 80% of microalbuminuric and macroalbuminuric patients, respectively, showed podocyturia, whereas none of the controls or normoalbuminuric patients did (Nakamura et al., 2000b). That study found no correlation between AER and the number of urinary podocytes, and patients with chronic renal failure failed to show podocyturia. Interestingly, here an ACE inhibitor, trandolapril, was able to reduce podocyturia in T2DM patients during 2-months follow-up (Nakamura et al., 2000b). In another study 21% of microalbuminuric T2DM patients showed podocyturia, and when these patients were treated with an antiplatelet drug dilazep dihydrochloride for six months their AER and also the number of podocytes in the urine decreased (Nakamura et

al., 2000a). In lupus nephritis, podocyturia has been found in the active phase of the disease in patients showing proteinuria of over 500 mg/24 h (Nakamura et al., 2000c). Here, patients with stable kidney function and healthy controls did not show any podocalyxin-positive cells in the urine.

Altered nephrin expression in diabetes was first demonstrated by Aaltonen et al. using experimental streptozotocin rat model (Aaltonen et al., 2001). Nephrin mRNA was increased at 4 weeks after induction of diabetes and the same initial increase was also found in non-obese diabetic mice. Increase in nephrin expression in the pre-proteinuric stage has also been observed in the PAN model (Hosoyamada et al., 2005). When proteinuria progresses, nephrin expression has been found to be reduced both at the mRNA and protein level in experimental (Forbes, 2002; Kelly et al., 2002) and human studies (Doublier et al., 2003; Koop et al., 2003). One study showed that expression of extracellular nephrin was diminished in DN but intracellular expression remained comparable to that of controls, although the intracellular expression was diminished in sclerotic areas (Benigni et al., 2004). It has been shown that the lower the numbers of nephrin positive cells (Toyoda et al., 2004) and the lower the level of nephrin mRNA expression are (Langham et al., 2002) in the glomeruli, the higher the level of urinary AER is.

One third of T1DM patients in Study II showed urinary proteins that reacted with nephrin antisera. The criterion for nephrinuria in Study II was the presence of any protein band detectable with both antisera in Western blots. The most common protein sizes found were 32, 40 and 75 kDa. When the patients with nephrinuria were compared to patients without nephrinuria no differences were found in clinical characteristics, such as duration of diabetes, body mass index, blood pressure, use of RAS modifying drugs, smoking, AER, glycated hemoglobin and cholesterol levels (Study II). Study III showed that of the urinary proteins reacting with nephrin antisera the 75 kDa protein most probably represents a nephrin fragment. In Study III the same patients as in Study II (Cohort I) and another T1DM patient cohort with or without nephropathy (Cohort II) were analyzed for the presence of the 75 kDa urinary nephrin. In both cohorts 75 kDa nephrin was found most commonly in the normoalbuminuric and microalbuminuric patients, but it was not detectable in urine of healthy controls. This suggests that 75 kDa nephrin is found specifically in T1DM and that its expression ceases when nephropathy progresses and proteinuria increases. In Cohort I most of the nephrinuric patients were female, but we did not find any significant differences in clinical variables between patients positive or negative for 75 kDa nephrin in the two cohorts.

A splicing variant of nephrin, nephrin α, has been found at the mRNA level in human (Holthofer et al., 1999) and rat glomeruli (Ahola et al., 1999). This form lacks the transmembrane region thus allowing production of a soluble form of the protein but the protein product has not yet been identified. Both in Study II and III we did not find full-length nephrin in the urine of T1DM patients. In the rat PAN model a 166 kDa urinary nephrin has been found (Luimula et al., 2000a) (Aaltonen et al., 2001). In the studies of Aaltonen and of Luimula, an antiserum against the extracellular domain of nephrin was used. We used antisera against nephrin α and

these antisera have epitopes in the intra- and extracellular domains near the transmembrane region (unpublished results). It is possible that our antisera detected a degraded form of nephrin. Nephrin α has only been found at the mRNA level and a relatively short transcript of it has been demonstrated using primers spanning the transmembrane region of intact nephrin (Ahola et al., 1999; Holthofer et al., 1999), thus it is not known whether additional splicing occurs in its outermost extracellular part. The 75 kDa nephrin may represent the splicing variant of nephrin or a fragment of if. Podocytes may start to secrete or shed soluble nephrin or nephrin-like proteins as a physiological response to an altered glucose environment. It may also represent nephrin that had detached from podocytes and that was proteolytically degraded during passage through the tubuli. Diabetes itself may cause metabolic changes and increase local proteolytic activity in the glomerulus leading to degradation of nephrin. Interestingly, proteolytic cleavage of urinary albumin has been observed in diabetic patients (Osicka et al., 2000a). The 75 kDa nephrin may also represent a yet unidentified nephrin-like protein. The finding that 75 kDa urinary nephrin was more common in normoalbuminuric patients compared to micro- and macroalbuminuric patients supports the finding that podocytes and/or nephrin are excreted in the early phase of the disease in the same manner as in podocyturia. The 75 kDa nephrin may also derive from urinary exosomes, as podocin and podocalyxin have been found in these (Knepper, 2002; Pisitkun et al., 2004). Interestingly, the nephrin-like protein NEPH2 is shed from podocytes by the action of a metalloproteinase, and it can be visualized with antibodies against the extracellular, but not the intracellular domain, suggesting that only the extracellular part is shed (Gerke et al., 2004). This form of the protein has also been found in urine of healthy subjects (Gerke et al., 2004). It is possible that the nephrin observed in our studies represent shed extracellular nephrin since our antisera do also recognize an epitope in the extracellular part of nephrin near the transmembrane region (unpublished results). Interestingly, the 32 and 40 kDa proteins lost positivity in Western blotting when an antiserum absorbed with E. coli lysate was used. Polyclonal antisera raised against recombinant proteins produced in E. coli may react with bacterial proteins that contaminate the immunogen in spite of the purification processes. When analyzing urine, contamination of the samples with E. coli is possible, since this bacterium belongs to the normal flora of the urinary tract. However, with antiserum that was absorbed with the E. coli lysate, the full-sized glomerular nephrin and the 75 kDa nephrin remained reactive. These proteins became invisible when the nephrin immunogen alpha-435 was used for absorption, indicating that the antiserum detects nephrin.

What could be the mechanisms behind the changes in nephrin expression? An ACE inhibitor, perindopril, restores nephrin expression on human kidney biopsies in DN (Langham et al., 2002). It has also been shown that the AGE inhibitor aminoguanidine normalizes the decreased nephrin expression in a diabetic rat model (Davis et al., 2004), but in another study no effects on nephrin expression were observed (Kelly et al., 2002). It is known that RAS modifiers and aminoguanidine are able to reduce PKC activity in experimental diabetes (Osicka et al., 2000b) and that nephrin expression may

be modified through this pathway (Wang et al., 2001b). Furthermore, PKC knockout mice are protected against development of albuminuria in diabetes (Menne et al., 2004). The effect of RAS inhibition seems to be quite specific for nephrin expression, since calcium channel blockers were not able to restore nephrin expression whereas valsartan was (Davis et al., 2003a). Interestingly, vasoactive mechanisms other than angiotensin II may be important modulators of nephrin expression, since the vasopeptidase inhibitor omapatrilat was also able to restore nephrin expression in a diabetic rat model (Davis et al., 2003b). Vasopeptidase inhibitors simultaneously inhibit both ACE and neutral endopeptidase leading to decreased levels of vasoconstrictor effector molecules such as angiotensin II as well as an increase in the levels of vasodilatory agents such as atrial natriuretic peptide and bradykinin (Fournie-Zaluski et al., 1994). Mechanical strain also causes up-regulation of angiotensin II and the production of angiotensin II type 1 receptors in podocyte culture, and valsartan ameliorates stretch-induced apoptosis (Durvasula et al., 2004). In the same study added exogenous angiotensin II alone increased podocyte apoptosis. Over-expression of angiotensin II type 1 receptors in podocytes led to glomerulosclerosis in a transgenic rat model (Hoffmann et al., 2004). In our studies females showed more often nephrinuria than did males. The difference could be explained by the fact that androgens stimulate the systemic and local RAS (Kang and Miller, 2002) and this could lead to increased angiotensin II levels and then decreased expression of nephrin in diabetes. This could also be one cause for the male gender being a known risk factor for DN (Seliger et al., 2001).

The occurrence of urinary 75 kDa nephrin was lower in T1DM patients with more severe nephropathy compared to that of normoalbuminuric and microalbuminuric patients in both patient cohorts in Study III. When proteinuria and DN progresses the expression of 75 kDa nephrin ceases. The patients of Cohort II were followed for 7.8 years for progression of DN. We analyzed the progression of patients that were normoalbuminuric or microalbuminuric at baseline, since the macroalbuminuric patients already had overt nephropathy and no patients were positive for 75 kDa nephrin in that group. These preliminary results showed that 20% of progressors were nephrinuric as compared to 42% of non-progressors. It is conceivable that 75 kDa nephrin is a marker of slower progression of diabetic nephropathy, but the finding was not statistically significant and more patients are needed to answer this question.

Nephrin is found in very low amounts in the pancreatic islets of Langerhans (Palmen et al., 2001; Putaala et al., 2001). Beta cells (Palmen et al., 2001) and islet microendothelia (Zanone et al., 2005) are proposed to exhibit specific expression. The function of pancreatic nephrin is still not known, but it does not appear to have any major significance for insulin secretion as studied in CNF patients using OGTT (Kuusniemi et al., 2004). Circulating antibodies against nephrin have been found in a subset of T1DM patients (Aaltonen et al., 2003). In experimental models injection of nephrin antibodies caused massive proteinuria in rats (Orikasa et al., 1988) and decreased nephrin expression (Kawachi et al., 2000). Interestingly, some CNF patients show increased nephrin autoantibody titers during recurrence of nephrotic syndrome after trans-

plantation (Patrakka et al., 2002b; Wang et al., 2001a). It remains to be studied whether nephrin autoantibodies have real pathophysiologic importance for the development of DN.

6.3. Nephrin and insulin resistance

In Study IV we found a 100 kDa urinary protein that reacted with a nephrin antiserum in the urine of approximately one third of the offspring of T2DM patients. Healthy controls that did not have any first-degree relative with T2DM were all negative for this protein. The offspring of T2DM patients have an almost threefold increased risk of diabetes in comparison to the background population and insulin resistance is an early metabolic feature of the offspring (Eriksson et al., 1989). Thus even though the study subjects were relatively healthy and young they could be considered somewhat abnormal regarding their glucose and insulin metabolism. Thus it is interesting that they showed changes in urinary excretion of the 100 kDa nephrin. The strongly positive subjects were more insulin resistant than weakly positive and negative ones. We did not find any difference between the positive and negative groups with respect to AER. Generally, microalbuminuria is considered a characteristic of the metabolic syndrome (Haffner et al., 1993). However, microalbuminuria has (Nosadini et al., 1992) (Forsblom et al., 1995) and has not (Toft et al., 2002) been associated with insulin resistance.

There are no studies concerning insulin resistance and the presence of podocyte proteins in urine but one study showed that piogliotazone was able to reduce both AER and the number of urinary podocytes in T2DM patients (Nakamura et al., 2001). Piogliotazone is an insulin-sensitizing agent that reduces insulin resistance by activating the peroxisome proliferator activated receptor gamma (PPAR-gamma). This pathway could also alter nephrin expression. Doublier et al. found that angiotensin II caused rearrangement of the actin cytoskeleton and decreased nephrin expression in cultured podocytes (Doublier et al., 2003). This was accompanied by excretion of a 100 kDa protein reacting with a nephrin antibody against the extracellular domain but not with an antibody against the intracellular domain (Doublier et al., 2003). It is possible that this protein represents the 100 kDa nephrin found in the offspring of T2DM in Study IV, since the antiserum used also recognizes an epitope in the extracellular domain of nephrin. Doublier's study also showed that glycated albumin caused a decrease in nephrin expression in podocytes and this was mediated through RAGE. When this pathway was active there was no excretion of the 100 kDa nephrin. RAS is involved in insulin resistance since RAS blockade increases adiponectin concentrations in patients with hypertension (Furuhashi et al., 2003). Adiponectin is an adipocyte-derived protein that has been suggested to play an important role in insulin sensitivity (Furuhashi et al., 2003). Large intervention studies have shown that ACE inhibitors and angiotensin-receptor antagonists may prevent development of T2DM (Hansson et al., 1999; Julius et al., 2004; Lindholm et al., 2002; Yusuf et al., 2000). The mechanisms causing insulin resistance may also affect podocytes. Insulin resistant diabetic Zucker rats show progressive diabetic nephropathy with evidence of podocyte injury and cultured podocytes exposed to high glucose show hypertrophy (Hoshi et al., 2002). A genetic model of lipoatrophic diabetes

(A-ZIP/F-1 mice) also show DN that is associated with podocyte damage (Suganami et al., 2005).

What could be the source of 100 kDa urinary nephrin, and why do the offspring of T2DM patients show this protein and not 75kDa urinary nephrin? What could be the biological significance of the 75 kDa and 100 kDa nephrins? The hypotheses proposed for the 75 kDa nephrin are valid also for the 100 kDa nephrin. The subjects in Study IV were healthy offspring of T2DM patients whereas the T1DM patients in Studies II and III had a long history of severely disturbed glucose metabolism causing pathophysiologic post-translational protein modifications, and altered intracellular signaling pathways. It must be addressed that T2DM is a heterogeneous disease with different, yet unidentified subgroups due to polygenic inheritance and environmental factors affecting the disease development and the pre-diabetic stage. This may explain why the results for T1DM patients differ from those for offspring of T2DM patients. Furthermore, in studies of T1DM patients a sample from 24-h urine collection was used instead of timed overnight urine in Study IV for offspring of T2DM patients. The 24-h urine sample may have been stored for a longer time at room temperature compared to the timed overnight urine sample that was collected and quickly frozen in the morning when the metabolic studies were conducted. Degradation during storage may have caused the difference in size of the nephrin fragments detected. This may also explain the finding that in Study IV a larger, 185-200 kDa protein was observed in some samples. All the samples in Studies II-IV were frozen without centrifugation, preservatives or proteinase inhibitors. The nephrins may originate from cells or from the soluble fraction of urine. Further identification and characterization of the 100 kDa and 75 kDa urinary nephrins will be particularly important. Interestingly, a 100 kDa form of nephrin was also detected in glomerular lysate in Study IV. To clarify the source of the nephrin fragments in urine the proteins need to be characterized by proteomic methods including mass-spectrometry. Nephrin has been identified using mass-spectrometry in only one study, in which nephrin was purified from 100 rat kidneys and identified by sequencing three peptides (Topham et al., 1999). Thus the task is demanding and characterization of the urinary nephrin fragments is the topic for further studies.

7. CONCLUSIONS

Puromycin causes proteinuria in ApoE mice on a high fat diet with overt hypercholesterolemia, but not in ApoE mice on a normal mouse diet. Pretreatment with the lipid antioxidant probucol reduces serum cholesterol levels and proteinuria. The proteinuric mice show decreased expression of glomerular nephrin, which is accompanied by increased levels of lipid peroxidation adducts, MDA and 4-HNE. Probucol also normalized the expression of nephrin and lipid peroxidation adducts. Taken together the results suggest a role for cholesterol and lipid peroxidation in proteinuria and in nephrin expression.

Approximately one third of type 1 diabetic patients with or without nephropathy showed urinary proteins detectable with nephrin antisera. Female patients more often showed nephrinuria than male patients. None of the control subjects showed nephrinuria. Of the urinary proteins reacting with nephrin antisera the 75 kDa protein appears to be nephrin-derived fragment. The occurrence of 75 kDa nephrin is highest in normo- and microalbuminuric type 1 diabetic patients and decreases when nephropathy progresses. 20% of progressors and 42% or non-progressors were nephrinuric at baseline in 7.8 years follow-up ($p=0.282$). Whether 75 kDa urinary nephrin has true prognostic value for diabetic nephropathy requires further investigation with a larger number of patients.

Proteins detectable with nephrin antiserum are more often present in urine of offspring of type 2 diabetic patients than in that of controls. A 100 kDa nephrin fragment was associated with insulin resistance and with lower levels of non-oxidative glucose disposal. Whether this protein may serve as a marker of susceptibility for type 2 diabetes needs further investigation and follow-up.

It would be important to find new urinary markers for insulin resistance and for the metabolic syndrome that could be used for evaluating the risk of individuals to develop T2DM. Such markers would provide new information about the pathological processes causing diabetes and diabetic complications and they might also facilitate development of new drugs that could prevent the adverse effects of diabetes.

8. YLEISTIETEELLINEN YHTEENVETO SUOMEKSI

Nuoruustyypin ja erityisesti aikuistyypin sokeritautia sairastavien potilaiden määrä lisääntyy ympäri maailman. Tämä kehitys aiheuttaa haasteita sokeritaudin ja sen liitännäissairauksien aikaiselle toteamiselle. Noin 20-30% sokeritautipotilaista kehittää taudin edetessä munuaisvaurion, yleisen liitännäissairauden, jonka toteamiseen käytetään virtsan tärkeimmän valkuaisen, albumiinin, mittausta. Munuaisvaurion esivaiheessa albumiinia vuotaa pieniä määriä munaisen suodatuskalvon lävitse virtsaan ja tätä ilmiötä kutsutaan mikroalbuminuriaksi. Mikroalbuminuriavaiheessa osalla sokeritautipotilaista on jo pysyviä muutoksia munuaisissaan. Nefriini on tärkeä munuaisen rakennevalkuaisaine ja se muodostaa keskeisen osan toiminnallisesta munuaisen suodatuskalvosta. Kokeellisissa malleissa ja sokeritautipotilaiden munuaisnäytteissä on todettu nefriinin vähenevän sokeritaudin munuaisvaurion kehittyessä.

Korkea veren kolesterolipitoisuus, hyperkolesterolemia, on tunnettu riskitekijä munuaisvaurion etenemiselle ja hyperkolesterolemia löytyy useimmilta munuaisvauriopotilaista.

Aikuistyypin sokeritautipotilaiden lähisukulaisten tiedetään olevan suuremmassa riskissä sairastua sokeritautiin kuin muun väestön. Aikuistyypin sokeritaudin kehittymistä edeltää usein tila, jossa kudokset kuten lihakset ja rasvakudos eivät reagoikaan enää yhtä hyvin sokeriaineenvaihdunnassa tärkeälle hormonille, insuliinille. Tätä tilaa kutsutaan insuliiniresistenssiksi. Insuliini toimittaa veressä olevan sokerin kudosten soluihin ja mikäli kudokset eivät enää reagoi sille veren sokeripitoisuus nousee. Aikuistyypin sokeritautipotilaiden lähisukulaisten, mm. lasten, tiedetään olevan insuliiniresistentimpejä kuin muun väestön.

Tutkimuksen tarkoituksena oli selvittää miten hyperkolesterolemia vaikuttaa munuaisvaurion kehittymiseen käyttämällä kokeellista mallia, jossa munuaisvaurio saadaan aikaiseksi puromysiini-nimisellä aineella hiirillä, joilla on geneettisen muuntelun vuoksi normaalia korkeammat veren kolesteroliarvot. Tutkimuksessa mitattiin valkuaisvirtsaisuuden kehittymistä, nefriinin muutoksia munuaisissa, sekä rasvojen hapettumisen yhteydessä syntyviä yhdisteitä munuaisissa, sekä sitä, miten rasvojen hapettumista vähentävä aine, probukoli, vaikuttaa mallissa. Hiirille, joilla oli hyperkolesterolemia, kehittyi vaikeampi munuaisvaurio kuin hiirille, joilla oli alhaisemmat veren kolesterolitasot. Probukoli vähensi veren kolesterolipitoisuutta ja lievensi myös munuaisvauriota. Mallissa havaittuun munuaisvaurioon liittyi myös nefriinin vähentyminen sekä rasvojen hapettumisen yhteydessä syntyvien tuotteiden lisääntyminen munuaisessa.

Väitöstutkimuksessa selvitettiin edelleen löytyykö nuoruustyypin sokeritautipotilaiden virtsasta rakennevalkuaisaine nefriiniä tai sen pilkkoutumistuotteita ja voidaanko virtsan nefriiniä pitää munuaisvaurion kehittymistä ennustavana merkkiaineena. Virtsanäyte tutkittiin kahden tutkimuksen potilailta, joista osalla ei ollut vielä munuaisvauriota, osalla oli munuaisvaurion varhaisvaihetta kuvaava mikroalbuminuria ja osalla jo merkittävä munuaisvaurio valkuaisvirtsaisuudella. Näistä potilaista noin kolmasosalta löytyi valkuaisaineita virtsasta, jotka oli osoitettavissa nefriiniin sitoutuvalla vasta-aineella. Jatkotutkimuksessa kävi ilmi, että näistä valkuaisaineista 75 kDa -kokoinen kappale edusti mitä todennäköisimmin nefriiniä tai nefriinin kaltaista valku-

aisainetta. Tämä valkuaisaine löytyi useimmiten sokeritautipotilailta, joilla ei ollut vielä merkkejä alkavasta tai varsinaisesta munuaisvauriosta, kun taas tämän valkuaisaineen esiintyvyys oli vähäisempi, mikäli munuaisvaurio oli edennyt pitkälle. Osa potilaista kuului seurantatutkimukseen, jossa osoittautui, että potilaat, joilta 75 kDa -nefriini löytyi tutkimuksen alkuvaiheessa, eivät edenneet varsinaiseen munuaistautiin yhtä nopeasti kuin ne, joiden virtsassa tätä valkuaisainetta ei ollut.

Kolmantena tavoitteena oli selvittää löytyykö nefriiniä tai sen kaltaista valkuaisainetta aikuistyypin sokeritautipotilaiden lähisukulaisten virtsasta ja mikäli löytyy, liittyykö sen esiintyminen jotenkin heidän sokeriaineenvaihduntansa muutoksiin. Kävi ilmi, että 27% lähisukulaisista löytyi virtsasta 100 kDa -kokoinen valkuaisaine, jota ei ollut terveiden kontrollihenkilöiden virtsassa. Ne, joilta kyseistä valkuaisainetta virtsasta löytyi, olivat insuliiniresistentimpejä kuin ne, joilta sitä ei löytynyt.

Korkea veren kolesterolipitoisuus, eli hyperkolesterolemia, on selvästi myös altistava tekijä munuaisvauriolle, eikä vain munuaisvaurion seuraus. Hyperkolesterolemian altistamaan munuaisvaurioon liittyy rasvojen hapettuminen ja nefriinin vähentyminen munuaisissa. 75 kDa -nefriini voi olla merkki nuoruustyypin sokeritautipotilaan pienemmästä riskistä saada munuaisvaurio, mutta potilasaineiston koosta johtuen näitä tuloksia on pidettävä alustavina. Aikuistyypin sokeritautipotilaiden lähisukulaisilta löytynyt 100 kDa -nefriini voisi toimia insuliiniresistenssin merkkiaineena ja mahdollisesti myös ennustaa riskiä sairastua aikuistyypin sokeritautiin. On tärkeää löytää uusia merkkiaineita sokeritaudin varhaisvaiheen tunnistamiselle ja liitännäissairauksien kehittymisen ennustamiselle, jotta näiden sairauksien kehittymistä estävät elintapamuutokset ja/tai lääkitys voidaan aloittaa tehokkaasti mahdollisimman varhaisessa vaiheessa. Virtsan merkkiaineet tuovat uutta tietoa tautien ja niiden liitännäissairauksien kehittymisestä ja voivat siten auttaa jopa uusien lääkkeiden kehittämistä.

9. ACKNOWLEDGEMENTS

This thesis was carried out at the Department of Bacteriology and Immunology in Haartman Institute and in Biomedicum Helsinki, Finland, during years 2001-2005. I wish to thank the present Head of the department Professor Seppo Meri and the previous Head of the department Risto Renkonen for providing excellent working facilities and supporting scientific atmosphere.

I want to express my gratitude to my supervisor Docent Harry Holthöfer for the excellent guidance, support and inspiration during the years. He gave me the opportunity to participate in interesting and challenging projects. I felt that he trusted in me and gave me the amount of responsibility that I was able to carry. Conversations with him always made me feel better during the difficult times.

I am most grateful to Professor Ulf-Håkan Stenman and Docent Arno Hänninen for giving invaluable criticism and comments for improving my thesis. I greatly appreciate the time and energy that they sacrificed to my thesis.

My sincere thanks belong to the collaborators Per-Henrik Groop, Carol Forsblom, Markku Laakso, Pauli Karhapää, Katriina Aalto-Setälä, and to all other members of the FinnDiane Study Group and Markku Laakso's group in Kuopio.

I am very grateful to all previous and present members of our research group for teaching me so much about protein chemistry and for the happy times that we spent together in work and elsewhere. My special thanks go to Zhu-Zhu Cheng, Heidi Taipale, Kristiina Nokelainen and Marika Havana for their excellent work in the projects. Thanks to Elsa, Liisa, Tuula, Paula, Eija, Johanna, Petri, Pekka, Heikki, Pauliina, Juuso, Ansa, Pia-Mari, Mervi, Eva, Satu, Markus, Tiina, Eeva, Marja-Leena, Dmitry, Jarmo, Maija, Hanna, Niclas, Harri, Pia, Charlotta, Petra, Kaisa, Marja and Laura.

I want to thank the personnel in the Department of Bacteriology and in the Department of Immunology in HUSLAB. I owe my special thanks to Martti Vaara, Ilkka Seppälä and Aaro Miettinen for giving me the opportunity to work as a doctor in the field of clinical microbiology during these years. I want to express my warmest thanks to all my colleagues and co-workers in HUSLAB.

I wish to thank all the people of the Department of Bacteriology and Immunology in Haartman Institute. Special thanks to Pirkko Kokkonen for her friendship. The staff of Paloheinä health care center in Helsinki is also thanked, since due to the lovely working athmophere there I still had the strenght to write my thesis during evenings at home.

I also want to thank my friends: Tarja, Kirsi, Kati, Kaisa, Inka, Henriikka, Sharon, Marika, Ira, Sinikka, Anna-Maija and Saara. My most sincere thanks go to Minna Jänis for her friendship and support. We have many times trouble-shooted scientific and social problems together during the last years.

I owe my warmest thanks to my family. My parents, Sinikka and Matti, taught me their attitude towards work and life in a kind and supporting way. I thank my brother Simo, sister Satu, Pietari and brother-in-law Timo for their love and support. I also want to thank my aunts Leena and Eeva together with Anneli, Tero and Tiina for their support.

Finally, I express my greatest and warmest thanks to my Timo who has always been there for me and has given his love and support unselfishly. This thesis would not have been possible without you!

This work was supported by grants from the Finnish Cultural Foundation, the Research Foundation of Orion Corporation, the Finnish Medical Foundation, Munuaissäätiö and the University of Helsinki.

Helsinki, 16th of August 2005

10. REFERENCES

Aaltonen, P., P. Luimula, E. Astrom, T. Palmen, T. Gronholm, E. Palojoki, I. Jaakkola, H. Ahola, I. Tikkanen, and H. Holthofer, 2001, Changes in the expression of nephrin gene and protein in experimental diabetic nephropathy: Laboratory Investigation, v. 81, p. 1185-90.

Aaltonen, P., P. Tossavainen, A.-M. Teppo, A. Patari, P. Kulmala, M. Knip, and H. Holthofer, 2003, Circulating antibodies to nephrin, a common antigen of pancreas and kidney, in patients with type 1 diabetes: American Society of Nephrology Renal Week, p. SA-PO469.

Ahola, H., E. Heikkilä, E. Åström, M. Inagaki, I. Izawa, H. Pavenstädt, D. Kerjaschki, and H. Holthöfer, 2003, A novel protein, densin, expressed by glomerular podocytes: Journal of the American Society of Nephrology, v. 14, p. 1731-7.

Ahola, H., S. X. Wang, P. Luimula, M. L. Solin, L. B. Holzman, and H. Holthofer, 1999, Cloning and expression of the rat nephrin homolog: American Journal of Pathology, v. 155, p. 907-13.

Alberti, K. G., and P. Z. Zimmet, 1998, Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation.[see comment]: Diabetic Medicine., v. 15, p. 539-53.

Amore, A., G. Mazzucco, F. Cavallo, G. Forni, B. Gianoglio, M. Motta, L. Peruzzi, F. Novelli, M. G. Porcellini, G. Cesano, and R. Coppo, 1996, Adriamycin-induced proteinuria in nude mice: an immune-system-mediated toxic effect: Nephrology, Dialysis, Transplantation, v. 11, p. 1012-8.

Anonymous, 1993, The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group.[see comment]: New England Journal of Medicine, v. 329, p. 977-86.

Anonymous, 1996, Captopril reduces the risk of nephropathy in IDDM patients with microalbuminuria. The Microalbuminuria Captopril Study Group: Diabetologia, v. 39, p. 587-93.

Anonymous, 1998a, Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group.[see comment][erratum appears in Lancet 1998 Nov 7;352(9139):1557]: Lancet, v. 352, p. 854-65.

Anonymous, 1998b, Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group.[see comment][erratum appears in Lancet 1999 Aug 14;354(9178):602]: Lancet, v. 352, p. 837-53.

Baekkeskov, S., H. J. Aanstoot, S. Christgau, A. Reetz, M. Solimena, M. Cascalho, F. Folli, H. Richter-Olesen, P. De Camilli, and P. D. Camilli, 1990, Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase.[erratum appears in Nature 1990 Oct 25;347(6295):782 Note: Camilli PD[corrected to DeCamilli P]]: Nature., v. 347, p. 151-6.

Bains, R., P. N. Furness, and D. R. Critchley, 1997, A quantitative immunofluorescence study of glomerular cell adhesion proteins in proteinuric states: Journal of Pathology, v. 183, p. 272-80.

Balda, M. S., and K. Matter, 2000, Transmembrane proteins of tight junctions: Seminars in Cell & Developmental Biology, v. 11, p. 281-9.

Barletta, G. M., I. A. Kovari, R. K. Verma, D. Kerjaschki, and L. B. Holzman, 2003, Nephrin and Neph1 Co-localize at the Podocyte Foot Process Intercellular Junction and Form cis Hetero-oligomers: Journal of Biological Chemistry, v. 278, p. 19266-71.

Barnett, A. H., C. Eff, R. D. Leslie, and D. A. Pyke, 1981, Diabetes in identical twins. A study of 200 pairs: Diabetologia, v. 20, p. 87-93.

Beltcheva, O., P. Martin, U. Lenkkeri, and K. Tryggvason, 2001, Mutation spectrum in the nephrin gene (NPHS1) in congenital nephrotic syndrome: Human Mutation, v. 17, p. 368-73.

Benigni, A., E. Gagliardini, S. Tomasoni, M. Abbate, P. Ruggenenti, R. Kalluri, and G. Remuzzi, 2004, Selective impairment of gene expression and assembly of nephrin in human diabetic nephropathy: Kidney International, v. 65, p. 2193-200.

Benigni, A., S. Tomasoni, E. Gagliardini, C. Zoja, J. A. Grunkemeyer, R. Kalluri, and G. Remuzzi, 2001, Blocking

angiotensin ii synthesis/activity preserves glomerular nephrin in rats with severe nephrosis: Journal of the American Society of Nephrology, v. 12, p. 941-8.

Bertani, T., V. Gambara, and G. Remuzzi, 1996, Structural basis of diabetic nephropathy in microalbuminuric NIDDM patients: a light microscopy study: Diabetologia, v. 39, p. 1625-8.

Binder, C. J., H. Weiher, M. Exner, and D. Kerjaschki, 1999, Glomerular overproduction of oxygen radicals in Mpv17 gene-inactivated mice causes podocyte foot process flattening and proteinuria: A model of steroid-resistant nephrosis sensitive to radical scavenger therapy: American Journal of Pathology, v. 154, p. 1067-75.

Blanco, S., J. Bonet, D. Lopez, I. Casas, and R. Romero, 2005, ACE inhibitors improve nephrin expression in Zucker rats with glomerulosclerosis: Kidney International Supplement, v. 93.

Bonnet, F., M. E. Cooper, H. Kawachi, T. J. Allen, G. Boner, and Z. Cao, 2001, Irbesartan normalises the deficiency in glomerular nephrin expression in a model of diabetes and hypertension: Diabetologia., v. 44, p. 874-7.

Bottazzo, G. F., B. M. Dean, J. M. McNally, E. H. MacKay, P. G. Swift, and D. R. Gamble, 1985, In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis: New England Journal of Medicine, v. 313, p. 353-60.

Boute, N., O. Gribouval, S. Roselli, F. Benessy, H. Lee, A. Fuchshuber, K. Dahan, M. C. Gubler, P. Niaudet, and C. Antignac, 2000, NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome: Nature Genetics, v. 24, p. 349-54.

Breiteneder-Geleff, S., K. Matsui, A. Soleiman, P. Meraner, H. Poczewski, R. Kalt, G. Schaffner, and D. Kerjaschki, 1997, Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis: American Journal of Pathology, v. 151, p. 1141-52.

Brenner, B. M., M. E. Cooper, D. de Zeeuw, W. F. Keane, W. E. Mitch, H. H. Parving, G. Remuzzi, S. M. Snapinn, Z. Zhang, S. Shahinfar, and R. S. Investigators, 2001, Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy.[see comment]: New England Journal of Medicine, v. 345, p. 861-9.

Brownlee, M., 2001, Biochemistry and molecular cell biology of diabetic complications: Nature, v. 414, p. 813-20.

Brummendorf, T., and F. G. Rathjen, 1995, Cell adhesion molecules 1: immunoglobulin superfamily: Protein Profile, v. 2, p. 963-1108.

Caulfield, J. P., J. J. Reid, and M. G. Farquhar, 1976, Alterations of the glomerular epithelium in acute aminonucleoside nephrosis. Evidence for formation of occluding junctions and epithelial cell detachment: Lab Invest, v. 34, p. 43-59.

Chen, A., C. H. Wei, L. F. Sheu, S. L. Ding, and W. H. Lee, 1995, Induction of proteinuria by adriamycin or bovine serum albumin in the mouse: Nephron, v. 69, p. 293-300.

Chothia, C., and E. Y. Jones, 1997, The molecular structure of cell adhesion molecules: Annual Review of Biochemistry, v. 66, p. 823-62.

Collins, V. R., G. K. Dowse, C. F. Finch, P. Z. Zimmet, and A. W. Linnane, 1989, Prevalence and risk factors for micro- and macroalbuminuria in diabetic subjects and entire population of Nauru: Diabetes, v. 38, p. 1602-10.

Dahl, U., A. Sjodin, L. Larue, G. L. Radice, S. Cajander, M. Takeichi, R. Kemler, and H. Semb, 2002, Genetic dissection of cadherin function during nephrogenesis: Molecular & Cellular Biology., v. 22, p. 1474-87.

Dalla Vestra, M., A. Masiero, A. M. Roiter, A. Saller, G. Crepaldi, and P. Fioretto, 2003, Is podocyte injury relevant in diabetic nephropathy?: studies in patients with type 2 diabetes: Diabetes, v. 52, p. 1031-5.

Davis, B. J., Z. Cao, M. de Gasparo, H. Kawachi, M. E. Cooper, and T. J. Allen, 2003a, Disparate effects of angiotensin II antagonists and calcium channel blockers on albuminuria in experimental diabetes and hypertension: potential role of nephrin: Journal of Hypertension, v. 21, p. 209-216.

Davis, B. J., J. M. Forbes, M. C. Thomas, G. Jerums, W. C. Burns, H. Kawachi, T. J. Allen, and M. E. Cooper, 2004, Superior renoprotective effects of combination therapy with ACE and AGE inhibition in the diabetic spontaneously hypertensive rat: Diabetologia, v. 47, p. 89-97.

Davis, B. J., C. I. Johnston, L. M. Burrell, W. C. Burns, E. Kubota, Z. Cao, M. E. Cooper, and T. J. Allen, 2003b, Renoprotective effects of vasopeptidase inhibition in an experimental model of diabetic nephropathy: Diabetologia, v. 46, p. 961-71.

de Knijff, P., A. Kaptein, D. Boomsma, H. M. Princen, R. R. Frants, and L. M. Havekes, 1991, Apolipoprotein E polymorphism affects plasma levels of lipoprotein(a): Atherosclerosis, v. 90, p. 169-74.

DeFronzo, R. A., 1988, Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM: Diabetes., v. 37, p. 667-87.

Dekan, G., C. Gabel, and M. G. Farquhar, 1991, Sulfate contributes to the negative charge of podocalyxin, the major sialoglycoprotein of the glomerular filtration slits: Proceedings of the National Academy of Sciences of the United States of America, v. 88, p. 5398-402.

Diamond, J. R., J. V. Bonventre, and M. J. Karnovsky, 1986, A role for oxygen free radicals in aminonucleoside nephrosis: Kidney Int, v. 29, p. 478-83.

Dianzani, M. U., 2003, 4-hydroxynonenal from pathology to physiology: Molecular Aspects of Medicine, v. 24, p. 263-72.

Doi, T., M. Hattori, L. Y. Agodoa, T. Sato, H. Yoshida, L. J. Striker, and G. E. Striker, 1990, Glomerular lesions in non-obese diabetic mouse: before and after the onset of hyperglycemia: Laboratory Investigation, v. 63, p. 204-12.

Donoviel, D. B., D. D. Freed, H. Vogel, D. G. Potter, E. Hawkins, J. P. Barrish, B. N. Mathur, C. A. Turner, R. Geske, C. A. Montgomery, M. Starbuck, M. Brandt, A. Gupta, R. Ramirez-Solis, B. P. Zambrowicz, and D. R. Powell, 2001, Proteinuria and perinatal lethality in mice lacking neph1, a novel protein with homology to nephrin: Molecular & Cellular Biology, v. 21, p. 4829-36.

Doublier, S., G. Salvidio, E. Lupia, V. Ruotsalainen, D. Verzola, G. Deferrari, and G. Camussi, 2003, Nephrin expression is reduced in human diabetic nephropathy: evidence for a distinct role for glycated albumin and angiotensin II: Diabetes, v. 52, p. 1023-30.

Doyonnas, R., D. B. Kershaw, C. Duhme, H. Merkens, S. Chelliah, T. Graf, and K. M. McNagny, 2001, Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin: Journal of Experimental Medicine, v. 194, p. 13-27.

Drenckhahn, D., and R. P. Franke, 1988, Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man: Laboratory Investigation, v. 59, p. 673-82.

Du, X. L., D. Edelstein, L. Rossetti, I. G. Fantus, H. Goldberg, F. Ziyadeh, J. Wu, and M. Brownlee, 2000, Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation: Proceedings of the National Academy of Sciences of the United States of America, v. 97, p. 12222-6.

Durvasula, R. V., A. T. Petermann, K. Hiromura, M. Blonski, J. Pippin, P. Mundel, R. Pichler, S. Griffin, W. G. Couser, and S. J. Shankland, 2004, Activation of a local tissue angiotensin system in podocytes by mechanical strain 1: Kidney International January, v. 65, p. 30-39.

Eckel, R. H., S. M. Grundy, and P. Z. Zimmet, 2005, The metabolic syndrome: Lancet, v. 365, p. 1415-28.

Endlich, N., K. R. Kress, J. Reiser, D. Uttenweiler, W. Kriz, P. Mundel, and K. Endlich, 2001, Podocytes respond to mechanical stress in vitro: Journal of the American Society of Nephrology, v. 12, p. 413-22.

Eriksson, J., A. Franssila-Kallunki, A. Ekstrand, C. Saloranta, E. Widen, C. Schalin, and L. Groop, 1989, Early metabolic defects in persons at increased risk for non-insulin-dependent diabetes mellitus: New England Journal of Medicine., v. 321, p. 337-43.

Fahrig, T., C. Landa, P. Pesheva, K. Kuhn, and M. Schachner, 1987, Characterization of binding properties of the myelin-associated glycoprotein to extracellular matrix constituents: EMBO Journal., v. 6, p. 2875-83.

Fan, Q., J. Ding, J. Zhang, N. Guan, and J. Deng, 2004, Effect of the knockdown of podocin mRNA on nephrin and alpha-actinin in mouse podocyte: Experimental Biology & Medicine, v. 229, p. 964-70.

Ferrara, N., 2002, VEGF and the quest for tumour angiogenesis factors: Nature Reviews. Cancer., v. 2, p. 795-803.

Forbes, J. M., 2002, Modulation of nephrin in the diabetic kidney: association with systemic hypertension and increasing albuminuria: Journal of Hypertension, v. 20, p. 985-992.

Forsblom, C. M., J. G. Eriksson, A. V. Ekstrand, A. M. Teppo, M. R. Taskinen, and L. C. Groop, 1995, Insulin resistance and abnormal albumin excretion in non-diabetic first-degree relatives of patients with NIDDM: Diabetologia., v. 38, p. 363-9.

Foster, R. R., M. A. Saleem, P. W. Mathieson, D. O. Bates, and S. J. Harper, 2005, Vascular endothelial growth factor and nephrin interact and reduce apoptosis in human podocytes: American Journal of Physiology Renal Fluid & Electrolyte Physiology, v. 288.

Fournie-Zaluski, M. C., W. Gonzalez, S. Turcaud, I. Pham, B. P. Roques, and J. B. Michel, 1994, Dual inhibition of angiotensin-converting enzyme and neutral endopeptidase by the orally active inhibitor mixanpril: a potential therapeutic approach in hypertension: Proceedings of the National Academy of Sciences of the United States of America, v. 91, p. 4072-6.

Frayling, T. M., J. C. Evans, M. P. Bulman, E. Pearson, L. Allen, K. Owen, C. Bingham, M. Hannemann, M. Shepherd, S. Ellard, and A. T. Hattersley, 2001, Beta-cell genes and diabetes: molecular and clinical characterization of mutations in transcription factors: Diabetes, v. 50, p. S94-100.

Friedewald, W. T., R. I. Levy, and D. S. Fredrickson, 1972, Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge: Clin Chem, v. 18, p. 499-502.

Fukami, K., S. Ueda, S. Yamagishi, S. Kato, Y. Inagaki, M. Takeuchi, Y. Motomiya, R. Bucala, S. Iida, K. Tamaki, T. Imaizumi, M. E. Cooper, and S. Okuda, 2004, AGEs activate mesangial TGF-beta-Smad signaling via an angiotensin II type I receptor interaction: Kidney International, v. 66, p. 2137-47.

Furuhashi, M., N. Ura, K. Higashiura, H. Murakami, M. Tanaka, N. Moniwa, D. Yoshida, and K. Shimamoto, 2003, Blockade of the renin-angiotensin system increases adiponectin concentrations in patients with essential hypertension: Hypertension., v. 42, p. 76-81.

Furukawa, T., S. Ohno, H. Oguchi, K. Hora, S. Tokunaga, and S. Furuta, 1991, Morphometric study of glomerular slit diaphragms fixed by rapid-freezing and freeze-substitution: Kidney International, v. 40, p. 621-4.

Gambara, V., G. Mecca, G. Remuzzi, and T. Bertani, 1993, Heterogeneous nature of renal lesions in type II diabetes: Journal of the American Society of Nephrology, v. 3, p. 1458-66.

Gepts, W., 1965, Pathologic anatomy of the pancreas in juvenile diabetes mellitus: Diabetes, v. 14, p. 619-33.

Gerke, P., T. B. Huber, L. Sellin, T. Benzing, and G. Walz, 2003, Homodimerization and Heterodimerization of the Glomerular Podocyte Proteins Nephrin and NEPH1: Journal of the American Society of Nephrology, v. 14, p. 918-26.

Gerke, P., L. Sellin, T. B. Huber, T. Benzing, and G. Walz, 2004, NEPH2 interacts with nephrin but not with NEPH1 and is cleaved from podocytes by metalloproteinases: ASN 2004.

Glassock, R., S. Adler, H. Ward, and A. Cohen, 1991, Primary glomerular disease, *in* B. Brenner, and F. Rector, eds., The Kidney: Philadelphia, W.B. Saunders company, p. 1182-1368.

Goldenberg, A., L. H. Ngoc, M. C. Thouret, V. Cormier-Daire, M. F. Gagnadoux, D. Chretien, C. Lefrancois, V. Geromel, A. Rotig, P. Rustin, A. Munnich, V. Paquis, C. Antignac, M. C. Gubler, P. Niaudet, P. de Lonlay, and E. Berard, 2005, Respiratory chain deficiency presenting as congenital nephrotic syndrome: Pediatric Nephrology, v. 20, p. 465-9.

Griendling, K. K., C. A. Minieri, J. D. Ollerenshaw, and R. W. Alexander, 1994, Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells: Circulation Research, v. 74, p. 1141-8.

Groop, P. H., T. Elliott, A. Ekstrand, A. Franssila-Kallunki, R. Friedman, G. C. Viberti, and M. R. Taskinen, 1996, Multiple lipoprotein abnormalities in type I diabetic patients with renal disease: Diabetes, v. 45, p. 974-9.

Gueler, F., S. Rong, J. K. Park, A. Fiebeler, J. Menne, M. Elger, D. N. Mueller, F. Hampich, R. Dechend, U. Kunter, F. C. Luft, and H. Haller, 2002, Postischemic acute renal failure is reduced by short-term statin treatment in a rat model: Journal of the American Society of Nephrology, v. 13, p. 2288-98.

Guez, S., M. Giani, M. L. Melzi, C. Antignac, and B. M. Assael, 1998, Adequate clinical control of congenital nephrotic syndrome by enalapril: Pediatric Nephrology, v. 12, p. 130-2.

Guyton, A. C., 1991, Formation of Urine by the Kidney, Textbook of Medical Physiology, W.B. Saunders company, p. 286-297.

Gwinner, W., U. Landmesser, R. P. Brandes, B. Kubat, J. Plasger, O. Eberhard, K. M. Koch, and C. J. Olbricht, 1997, Reactive oxygen species and antioxidant defense in puromycin aminonucleoside glomerulopathy: J Am Soc Nephrol, v. 8, p. 1722-31.

Haffner, S. M., C. Gonzales, R. A. Valdez, L. Mykkanen, H. P. Hazuda, B. D. Mitchell, A. Monterrosa, and M. P. Stern, 1993, Is microalbuminuria part of the prediabetic state? The Mexico City Diabetes Study: Diabetologia., v. 36, p. 1002-6.

Haffner, S. M., M. P. Stern, B. D. Mitchell, H. P. Hazuda, and J. K. Patterson, 1990, Incidence of type II diabetes in Mexican Americans predicted by fasting insulin and glucose levels, obesity, and body-fat distribution: Diabetes., v. 39, p. 283-8.

Haffner, S. M., R. A. Valdez, H. P. Hazuda, B. D. Mitchell, P. A. Morales, and M. P. Stern, 1992, Prospective analysis of the insulin-resistance syndrome (syndrome X): Diabetes., v. 41, p. 715-22.

Hallman, N., L. Hjelt, and E. K. Ahvenainen, 1956, Nephrotic syndrome in newborn and young infants: Annales Paediatriae Fenniae., v. 2, p. 227-41.

Hamano, Y., J. A. Grunkemeyer, A. Sudhakar, M. Zeisberg, D. Cosgrove, R. Morello, B. Lee, H. Sugimoto, and R. Kalluri, 2002, Determinants of vascular permeability in the kidney glomerulus: Journal of Biological Chemistry, v. 277, p. 31154-62.

Hanley, A. J., K. Williams, C. Gonzalez, R. B. D'Agostino, Jr., L. E. Wagenknecht, M. P. Stern, S. M. Haffner, S. San Antonio Heart, S. Mexico City Diabetes, and S. Insulin Resistance Atherosclerosis, 2003, Prediction of type 2 diabetes using simple measures of insulin resistance: combined results from the San Antonio Heart Study, the Mexico City Diabetes Study, and the Insulin Resistance Atherosclerosis Study.: Diabetes., v. 52, p. 463-9.

Hanninen, A., S. Jalkanen, M. Salmi, S. Toikkanen, G. Nikolakaros, and O. Simell, 1992, Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus: Journal of Clinical Investigation, v. 90, p. 1901-10.

Hansson, L., L. H. Lindholm, L. Niskanen, J. Lanke, T. Hedner, A. Niklason, K. Luomanmaki, B. Dahlof, U. de Faire, C. Morlin, B. E. Karlberg, P. O. Wester, and J. E. Bjorck, 1999, Effect of angiotensin-converting-enzyme inhibition compared with conventional therapy on cardiovascular morbidity and mortality in hypertension: the Captopril Prevention Project (CAPPP) randomised trial.[see comment]: Lancet., v. 353, p. 611-6.

Harris, M. I., W. C. Hadden, W. C. Knowler, and P. H. Bennett, 1987, Prevalence of diabetes and impaired glucose tolerance and plasma glucose levels in U.S. population aged 20-74 yr: Diabetes, v. 36, p. 523-34.

Harris, M. I., R. Klein, T. A. Welborn, and M. W. Knuiman, 1992, Onset of NIDDM occurs at least 4-7 yr before clinical diagnosis: Diabetes Care., v. 15, p. 815-9.

Hawkins, N. J., D. Wakefield, and J. A. Charlesworth, 1990, The role of mesangial cells in glomerular pathology: Pathology, v. 22, p. 24-32.

Hiltunen, M., H. Hyoty, M. Knip, J. Ilonen, H. Reijonen, P. Vahasalo, M. Roivainen, M. Lonnrot, P. Leinikki, T. Hovi, and H. K. Akerblom, 1997, Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group: Journal of Infectious Diseases, v. 175, p. 554-60.

Hirano, T., J. C. Mamo, S. Nagano, and T. Sugisaki, 1991, The lowering effect of probucol on plasma lipoprotein and proteinuria in puromycin aminonucleoside-induced nephrotic rats: Nephron, v. 58, p. 95-100.

Hoffmann, S., D. Podlich, H. A. B, W. Kriz, and N. Gretz, 2004, Angiotensin II Type 1 Receptor Overexpression in Podocytes Induces Glomerulosclerosis in Transgenic Rats: Journal of the American Society of Nephrology, v. 15, p. 1475-87.

Holmberg, C., J. Laine, K. Ronnholm, M. Ala-Houhala, and H. Jalanko, 1996, Congenital nephrotic syndrome: Kidney International Supplement, v. 53.

Holthofer, H., H. Ahola, M. L. Solin, S. Wang, T. Palmen, P. Luimula, A. Miettinen, and D. Kerjaschki, 1999, Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney: Am J Pathol, v. 155, p. 1681-7.

Holthofer, H., J. Reivinen, and A. Miettinen, 1994, Nephron segment and cell-type specific expression of gangliosides in the developing and adult kidney: Kidney Int, v. 45, p. 123-30.

Holzman, L. B., P. L. St John, I. A. Kovari, R. Verma, H. Holthofer, and D. R. Abrahamson, 1999, Nephrin localizes to the slit pore of the glomerular epithelial cell: Kidney International, v. 56, p. 1481-91.

Hoshi, S., Y. Shu, F. Yoshida, T. Inagaki, J. Sonoda, T. Watanabe, K. Nomoto, and M. Nagata, 2002, Podocyte injury promotes progressive nephropathy in zucker diabetic fatty rats: Laboratory Investigation, v. 82, p. 25-35.

Hosoyamada, M., K. Yan, Y. Nishibori, Y. Takiue, A. Kudo, H. Kawakami, T. Shibasaki, and H. Endou, 2005, Nephrin and podocin expression around the onset of puromycin aminonucleoside nephrosis: Journal of Pharmacological Sciences, v. 97, p. 234-41.

Hostetter, T. H., H. G. Rennke, and B. M. Brenner, 1982, The case for intrarenal hypertension in the initiation and progression of diabetic and other glomerulopathies: American Journal of Medicine, v. 72, p. 375-80.

Huber, T. B., B. Hartleben, J. Kim, M. Schmidts, B. Schermer, A. Keil, L. Egger, R. L. Lecha, C. Borner, H. PavenstAdt, A. S. Shaw, G. Walz, and T. Benzing, 2003a, Nephrin and CD2AP Associate with Phosphoinositide 3-OH Kinase and Stimulate AKT-Dependent Signaling: Molecular & Cellular Biology, v. 23, p. 4917-28.

Huber, T. B., M. Kottgen, B. Schilling, G. Walz, and T. Benzing, 2001, Interaction with podocin facilitates nephrin signaling: Journal of Biological Chemistry., v. 276, p. 41543-6.

Huber, T. B., M. Simons, B. Hartleben, L. Sernetz, M. Schmidts, E. Gundlach, M. A. Saleem, G. Walz, and T. Benzing, 2003b, Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains: Human Molecular Genetics, v. 12, p. 3397-405.

Hui, D. Y., T. L. Innerarity, and R. W. Mahley, 1981, Lipoprotein binding to canine hepatic membranes. Metabolically distinct apo-E and apo-B,E receptors: Journal of Biological Chemistry, v. 256, p. 5646-55.

Huttunen, N. P., J. Rapola, J. Vilska, and N. Hallman, 1980, Renal pathology in congenital nephrotic syndrome of Finnish type: a quantitative light microscopic study on 50 patients: International Journal of Pediatric Nephrology, v. 1, p. 10-6.

Huwiler, A., S. Ren, H. Holthofer, H. Pavenstadt, and J. Pfeilschifter, 2003, Inflammatory cytokines upregulate nephrin expression in human embryonic kidney epithelial cells and podocytes: Biochemical & Biophysical Research Communications, v. 305, p. 136-42.

Hyoty, H., M. Hiltunen, M. Knip, M. Laakkonen, P. Vahasalo, J. Karjalainen, P. Koskela, M. Roivainen, P. Leinikki, T. Hovi, and et al., 1995, A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group: Diabetes, v. 44, p. 652-7.

Ihalmo, P., T. Palmen, H. Ahola, E. Valtonen, and H. Holthofer, 2003, Filtrin is a novel member of nephrin-like proteins: Biochemical & Biophysical Research Communications, v. 300, p. 364-70.

Inman, S. R., N. T. Stowe, M. D. Cressman, B. H. Brouhard, J. V. Nally, Jr., S. Satoh, R. Satodate, and D. G. Vidt, 1999, Lovastatin preserves renal function in experimental diabetes: American Journal of the Medical Sciences, v. 317, p. 215-21.

Inoue, T., E. Yaoita, H. Kurihara, F. Shimizu, T. Sakai, T. Kobayashi, K. Ohshiro, H. Kawachi, H. Okada, H. Suzuki, I. Kihara, and T. Yamamoto, 2001, FAT is a component of glomerular slit diaphragms: Kidney International, v. 59, p. 1003-12.

Itoh, N., T. Hanafusa, A. Miyazaki, J. Miyagawa, K. Yamagata, K. Yamamoto, M. Waguri, A. Imagawa, S. Tamura, M. Inada, and et al., 1993, Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients: Journal of Clinical Investigation, v. 92, p. 2313-22.

Jennette, J., 2004, Pathology of diabetic glomerulosclerosis, *in* J. A. Bruijn, and A. B. Fogo, eds., Postgraduate education course, Renal Week 2004: St. Louis, Missouri, The American Society of Nephrology, p. 53-54.

Jensen, T., S. Stender, and T. Deckert, 1988, Abnormalities in plasmas concentrations of lipoproteins and fibrinogen in type 1 (insulin-dependent) diabetic patients with increased urinary albumin excretion: Diabetologia, v. 31, p. 142-5.

Julius, S., S. E. Kjeldsen, M. Weber, H. R. Brunner, S. Ekman, L. Hansson, T. Hua, J. Laragh, G. T. McInnes, L. Mitchell, F. Plat, A. Schork, B. Smith, A. Zanchetti, and V. t. group, 2004, Outcomes in hypertensive patients at high cardiovascular risk treated with regimens based on valsartan or amlodipine: the VALUE randomised trial: Lancet, v. 363, p. 2022-31.

Junod, A., A. E. Lambert, L. Orci, R. Pictet, A. E. Gonet, and A. E. Renold, 1967, Studies of the diabetogenic action of streptozotocin: Proceedings of the Society for Experimental Biology & Medicine, v. 126, p. 201-5.

Kang, A. K., and J. A. Miller, 2002, Effects of gender on the renin-angiotensin system, blood pressure, and renal function: Current Hypertension Reports, v. 4, p. 143-51.

Kaplan, J. M., S. H. Kim, K. N. North, H. Rennke, L. A. Correia, H. Q. Tong, B. J. Mathis, J. C. Rodriguez-Perez, P. G. Allen, A. H. Beggs, and M. R. Pollak, 2000, Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis: Nature Genetics, v. 24, p. 251-6.
Karle, S. M., B. Uetz, V. Ronner, L. Glaeser, F. Hildebrandt, and A. Fuchshuber, 2002, Novel mutations in NPHS2 de-

tected in both familial and sporadic steroid-resistant nephrotic syndrome: Journal of the American Society of Nephrology., v. 13, p. 388-93.

Kasiske, B. L., M. P. O'Donnell, W. J. Garvis, and W. F. Keane, 1988, Pharmacologic treatment of hyperlipidemia reduces glomerular injury in rat 5/6 nephrectomy model of chronic renal failure: Circulation Research, v. 62, p. 367-74.

Kawachi, H., H. Koike, H. Kurihara, T. Sakai, and F. Shimizu, 2003, Cloning of rat homologue of podocin: expression in proteinuric states and in developing glomeruli: Journal of the American Society of Nephrology, v. 14, p. 46-56.

Kawachi, H., H. Koike, H. Kurihara, E. Yaoita, M. Orikasa, M. A. Shia, T. Sakai, T. Yamamoto, D. J. Salant, and F. Shimizu, 2000, Cloning of rat nephrin: expression in developing glomeruli and in proteinuric states: Kidney Int, v. 57, p. 1949-61.

Kawachi, H., H. Kurihara, P. S. Topham, D. Brown, M. A. Shia, M. Orikasa, F. Shimizu, and D. J. Salant, 1997, Slit diaphragm-reactive nephritogenic MAb 5-1-6 alters expression of ZO-1 in rat podocytes: American Journal of Physiology, v. 273, p. F984-93.

Kawaguchi, M., M. Yamada, H. Wada, and T. Okigaki, 1992, Roles of active oxygen species in glomerular epithelial cell injury in vitro caused by puromycin aminonucleoside.: Toxicology, v. 72, p. 329-340.

Kaysen, G. A., B. D. Myers, W. G. Couser, R. Rabkin, and J. M. Felts, 1986, Mechanisms and consequences of proteinuria: Lab Invest, v. 54, p. 479-98.

Keane, W. F., B. L. Kasiske, and M. P. O'Donnell, 1988, Lipids and progressive glomerulosclerosis. A model analogous to atherosclerosis: Am J Nephrol, v. 8, p. 261-71.

Kelly, D. J., P. Aaltonen, A. J. Cox, J. R. Rumble, R. Langham, S. Panagiotopoulos, G. Jerums, H. Holthofer, and R. E. Gilbert, 2002, Expression of the slit-diaphragm protein, nephrin, in experimental diabetic nephropathy: differing effects of anti-proteinuric therapies: Nephrology Dialysis Transplantation, v. 17, p. 1327-32.

Kerjaschki, D., 2001, Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis: Journal of Clinical Investigation., v. 108, p. 1583-7.

Kerjaschki, D., M. Exner, R. Ullrich, M. Susani, L. K. Curtiss, J. L. Witztum, M. G. Farquhar, and R. A. Orlando, 1997, Pathogenic antibodies inhibit the binding of apolipoproteins to megalin/gp330 in passive Heymann nephritis: Journal of Clinical Investigation, v. 100, p. 2303-9.

Kerjaschki, D., and M. G. Farquhar, 1983, Immunocytochemical localization of the Heymann nephritis antigen (GP330) in glomerular epithelial cells of normal Lewis rats: Journal of Experimental Medicine, v. 157, p. 667-86.

Kerjaschki, D., P. P. Ojha, M. Susani, R. Horvat, S. Binder, A. Hovorka, P. Hillemanns, and R. Pytela, 1989, A beta 1-integrin receptor for fibronectin in human kidney glomeruli: American Journal of Pathology, v. 134, p. 481-9.

Kerjaschki, D., D. J. Sharkey, and M. G. Farquhar, 1984, Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell: Journal of Cell Biology., v. 98, p. 1591-6.

Kerosuo, L., E. Juvonen, R. Alitalo, M. Gylling, D. Kerjaschki, and A. Miettinen, 2004, Podocalyxin in human haematopoietic cells: British Journal of Haematology., v. 124, p. 809-18.

Kestila, M., U. Lenkkeri, M. Mannikko, J. Lamerdin, P. McCready, H. Putaala, V. Ruotsalainen, T. Morita, M. Nissinen, R. Herva, C. E. Kashtan, L. Peltonen, C. Holmberg, A. Olsen, and K. Tryggvason, 1998, Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome: Mol Cell, v. 1, p. 575-82.

Kestila, M., M. Mannikko, C. Holmberg, G. Gyapay, J. Weissenbach, E. R. Savolainen, L. Peltonen, and K. Tryggvason, 1994, Congenital nephrotic syndrome of the Finnish type maps to the long arm of chromosome 19: American Journal of Human Genetics, v. 54, p. 757-64.

Khoshnoodi, J., K. Sigmundsson, L. G. Ofverstedt, U. Skoglund, B. Obrink, J. Wartiovaara, and K. Tryggvason, 2003, Nephrin Promotes Cell-Cell Adhesion through Homophilic Interactions: American Journal of Pathology, v. 163, p. 2337-46.

Kim, Y. H., M. Goyal, D. Kurnit, B. Wharram, J. Wiggins, L. Holzman, D. Kershaw, and R. Wiggins, 2001, Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat: Kidney International, v. 60, p. 957-68.

Kimmelstiel, P., and C. Wilson, 1936, Intercapillary lesions in the glomeruli of the kidney.: Am J Pathol, v. 12, p. 83-105.

Kimpimaki, T., M. Erkkola, S. Korhonen, A. Kupila, S. M. Virtanen, J. Ilonen, O. Simell, and M. Knip, 2001a, Short-term exclusive breastfeeding predisposes young children with increased genetic risk of Type I diabetes to progressive beta-cell autoimmunity: Diabetologia, v. 44, p. 63-9.

Kimpimaki, T., A. Kupila, A. M. Hamalainen, M. Kukko, P. Kulmala, K. Savola, T. Simell, P. Keskinen, J. Ilonen, O. Simell, and M. Knip, 2001b, The first signs of beta-cell autoimmunity appear in infancy in genetically susceptible children from the general population: the Finnish Type 1 Diabetes Prediction and Prevention Study: Journal of Clinical Endocrinology & Metabolism, v. 86, p. 4782-8.

Knepper, M. A., 2002, Proteomics and the kidney: J Am Soc Nephrol, v. 13, p. 1398-408.

Knepper, M. A., 2004, Proteomic Studies of Aquaporin-2 Regulation in the Collection Duct: 40th Annual Scientific Meeting of the Australian and New Zealand Society of Nephrology.

Knip, M., 2002, Can we predict type 1 diabetes in the general population?: Diabetes Care, v. 25, p. 623-625.

Kolm-Litty, V., U. Sauer, A. Nerlich, R. Lehmann, and E. D. Schleicher, 1998, High glucose-induced transforming growth factor beta1 production is mediated by the hexosamine pathway in porcine glomerular mesangial cells: Journal of Clinical Investigation, v. 101, p. 160-9.

Koop, K., M. Eikmans, H. J. Baelde, H. Kawachi, E. De Heer, L. C. Paul, and J. A. Bruijn, 2003, Expression of podocyte-associated molecules in acquired human kidney diseases: Journal of the American Society of Nephrology, v. 14, p. 2063-71.

Koya, D., M. R. Jirousek, Y. W. Lin, H. Ishii, K. Kuboki, and G. L. King, 1997, Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats: Journal of Clinical Investigation, v. 100, p. 115-26.

Koya, D., and G. L. King, 1998, Protein kinase C activation and the development of diabetic complications.: Diabetes, v. 47, p. 859-866.

Koziell, A., V. Grech, S. Hussain, G. Lee, U. Lenkkeri, K. Tryggvason, and P. Scambler, 2002, Genotype/phenotype correlations of *NPHS1* and *NPHS2* mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration: Human Molecular Genetics, v. 11, p. 379-388.

Kreidberg, J. A., M. J. Donovan, S. L. Goldstein, H. Rennke, K. Shepherd, R. C. Jones, and R. Jaenisch, 1996, Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis: Development - Supplement, v. 122, p. 3537-47.

Kriz, W., N. Gretz, and K. V. Lemley, 1998, Progression of glomerular diseases: Is the podocyte the culprit?: Kidney International September, v. 54, p. 687-697.

Kriz, W., M. Kretzler, A. P. Provoost, and I. Shirato, 1996, Stability and leakiness: opposing challenges to the glomerulus: Kidney International, v. 49, p. 1570-4.

Kupila, A., P. Muona, T. Simell, P. Arvilommi, H. Savolainen, A. M. Hamalainen, S. Korhonen, T. Kimpimaki, M. Sjoroos, J. Ilonen, M. Knip, O. Simell, and I. D. i. F. Juvenile Diabetes Research Foundation Centre for the Prevention of Type, 2001, Feasibility of genetic and immunological prediction of type I diabetes in a population-based birth cohort: Diabetologia, v. 44, p. 290-7.

Kurihara, H., J. M. Anderson, and M. G. Farquhar, 1992a, Diversity among tight junctions in rat kidney: glomerular slit diaphragms and endothelial junctions express only one isoform of the tight junction protein ZO-1: Proceedings of the National Academy of Sciences of the United States of America, v. 89, p. 7075-9.

Kurihara, H., J. M. Anderson, D. Kerjaschki, and M. G. Farquhar, 1992b, The altered glomerular filtration slits seen in puromycin aminonucleoside nephrosis and protamine sulfate-treated rats contain the tight junction protein ZO-1: American Journal of Pathology, v. 141, p. 805-16.

Kuusniemi, A. M., M. Kestila, J. Patrakka, A. T. Lahdenkari, V. Ruotsalainen, C. Holmberg, R. Karikoski, R. Salonen, K. Tryggvason, and H. Jalanko, 2004, Tissue expression of nephrin in human and pig: Pediatric Research, v. 55, p. 774-81.

Lahdenperä, J., P. Kilpeläinen, X. L. Liu, T. Pikkarainen, P. Reponen, V. Ruotsalainen, and K. Tryggvason, 2003, Clustering-induced tyrosine phosphorylation of nephrin by Src family kinases: Kidney International, v. 64, p. 404-13.

Lan, M. S., C. Wasserfall, N. K. Maclaren, and A. L. Notkins, 1996, IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus: Proceedings of the National

Academy of Sciences of the United States of America., v. 93, p. 6367-70.

Langham, R. G., D. J. Kelly, A. J. Cox, N. M. Thomson, H. Holthofer, P. Zaoui, N. Pinel, D. J. Cordonnier, and R. E. Gilbert, 2002, Proteinuria and the expression of the podocyte slit diaphragm protein, nephrin, in diabetic nephropathy: effects of angiotensin converting enzyme inhibition: Diabetologia, v. 45, p. 1572-6.

Lassila, M., K. K. Seah, T. J. Allen, V. Thallas, M. C. Thomas, R. Candido, W. C. Burns, J. M. Forbes, A. C. Calkin, M. E. Cooper, and K. A. M. Jandeleit-Dahm, 2004, Accelerated Nephropathy in Diabetic Apolipoprotein E-Knockout Mouse: Role of Advanced Glycation End Products: Journal of the American Society of Nephrology August, v. 15, p. 2125-2138.

Lee, Y. K., T. Kwon, D. J. Kim, W. Huh, Y. G. Kim, H. Y. Oh, and H. Kawachi, 2004, Ultrastructural study on nephrin expression in experimental puromycin aminonucleoside nephrosis: Nephrology Dialysis Transplantation, v. 19, p. 2981-6.

Lehtonen, S., E. Lehtonen, K. Kudlicka, H. Holthofer, and M. G. Farquhar, 2004, Nephrin forms a complex with adherens junction proteins and CASK in podocytes and in Madin-Darby canine kidney cells expressing nephrin: American Journal of Pathology, v. 165, p. 923-36.

Lenkkeri, U., M. Mannikko, P. McCready, J. Lamerdin, O. Gribouval, P. M. Niaudet, C. K. Antignac, C. E. Kashtan, C. Homberg, A. Olsen, M. Kestila, and K. Tryggvason, 1999, Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations: American Journal of Human Genetics, v. 64, p. 51-61.

Lewis, E. J., L. G. Hunsicker, R. P. Bain, and R. D. Rohde, 1993, The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group.[comment][erratum appears in N Engl J Med 1993 Jan 13;330(2):152]: New England Journal of Medicine., v. 329, p. 1456-62.

Lewis, E. J., L. G. Hunsicker, W. R. Clarke, T. Berl, M. A. Pohl, J. B. Lewis, E. Ritz, R. C. Atkins, R. Rohde, I. Raz, and G. Collaborative Study, 2001, Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes.[comment]: New England Journal of Medicine., v. 345, p. 851-60.

Li, C., V. Ruotsalainen, K. Tryggvason, A. S. Shaw, and J. H. Miner, 2000, CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere: American Journal of Physiology - Renal Fluid & Electrolyte Physiology, v. 279, p. F785-92.

Liberopoulos, E., K. Siamopoulos, and M. Elisaf, 2004, Apolipoprotein E and renal disease: American Journal of Kidney Diseases, v. 43, p. 223-33.

Lillioja, S., D. M. Mott, J. K. Zawadzki, A. A. Young, W. G. Abbott, W. C. Knowler, P. H. Bennett, P. Moll, and C. Bogardus, 1987, In vivo insulin action is familial characteristic in nondiabetic Pima Indians: Diabetes, v. 36, p. 1329-35.

Lindholm, L. H., H. Ibsen, K. Borch-Johnsen, M. H. Olsen, K. Wachtell, B. Dahlof, R. B. Devereux, G. Beevers, U. de Faire, F. Fyhrquist, S. Julius, S. E. Kjeldsen, K. Kristianson, O. Lederballe-Pedersen, M. S. Nieminen, P. Omvik, S. Oparil, H. Wedel, P. Aurup, J. M. Edelman, S. Snapinn, and L. s. g. For the, 2002, Risk of new-onset diabetes in the Losartan Intervention For Endpoint reduction in hypertension study: Journal of Hypertension., v. 20, p. 1879-86.

Liu, G., B. Kaw, J. Kurfis, S. Rahmanuddin, Y. S. Kanwar, and S. S. Chugh, 2003, Neph1 and nephrin interaction in the slit diaphragm is an important determinant of glomerular permeability: Journal of Clinical Investigation, v. 112, p. 209-21.

Liu, L., K. Aya, H. Tanaka, J. Shimizu, S. Ito, and Y. Seino, 2001, Nephrin is an important component of the barrier system in the testis: Acta Medica Okayama, v. 55, p. 161-5.

Liu, X. L., P. Kilpelainen, U. Hellman, Y. Sun, J. Wartiovaara, E. Morgunova, T. Pikkarainen, K. Yan, A. P. Jonsson, and K. Tryggvason, 2005, Characterization of the interactions of the nephrin intracellular domain: FEBS Journal, v. 272, p. 228-43.

Ljungberg, P., H. Jalanko, C. Holmberg, and H. Holthofer, 1993, Congenital nephrosis of the Finnish type (CNF): matrix components of the glomerular basement membranes and of cultured mesangial cells: Histochemical Journal, v. 25, p. 606-12.

Luimula, P., P. Aaltonen, H. Ahola, T. Palmen, and H. Holthofer, 2000a, Alternatively spliced nephrin in experimental glomerular disease of the rat: Pediatric Research, v. 48, p. 759-62.

Luimula, P., H. Ahola, S. X. Wang, M. L. Solin, P. Aaltonen, I. Tikkanen, D. Kerjaschki, and H. Holthofer, 2000b, Nephrin in experimental glomerular disease: Kidney Int, v. 58, p. 1461-8.

Luimula, P., N. Sandstrom, D. Novikov, and H. Holthofer, 2002, Podocyte-associated molecules in puromycin aminonucleoside nephrosis of the rat: Laboratory Investigation, v. 82, p. 713-8.

Macconi, D., M. Ghilardi, M. E. Bonassi, E. I. Mohamed, M. Abbate, F. Colombi, G. Remuzzi, and A. Remuzzi, 2000, Effect of angiotensin-converting enzyme inhibition on glomerular basement membrane permeability and distribution of zonula occludens-1 in MWF rats: Journal of the American Society of Nephrology, v. 11, p. 477-89.

Mahley, R. W., 1986, The molecular basis of atherosclerosis: concepts derived from studies of lipoprotein metabolism and cell biology: Clinical & Investigative Medicine Medecine Clinique et Experimentale, v. 9, p. 304-8.

Mahley, R. W., and Y. Huang, 1999, Apolipoprotein E: from atherosclerosis to Alzheimer's disease and beyond.: Curr Opin Lipidol, v. 10, p. 207-217.

Mannikko, M., M. Kestaila, C. Holmberg, R. Norio, M. Ryynanen, A. Olsen, L. Peltonen, and K. Tryggvason, 1995, Fine mapping and haplotype analysis of the locus for congenital nephrotic syndrome on chromosome 19q13.1: American Journal of Human Genetics, v. 57, p. 1377-83.

Marnett, L. J., 2002, Oxy radicals, lipid peroxidation and DNA damage: Toxicology, v. 182, p. 219-22.

Matsui, K., S. Breiteneder-Geleff, and D. Kerjaschki, 1998, Epitope-specific antibodies to the 43-kD glomerular membrane protein podoplanin cause proteinuria and rapid flattening of podocytes: Journal of the American Society of Nephrology, v. 9, p. 2013-26.

Mauer, S. M., M. W. Steffes, E. N. Ellis, D. E. Sutherland, D. M. Brown, and F. C. Goetz, 1984, Structural-functional relationships in diabetic nephropathy: Journal of Clinical Investigation., v. 74, p. 1143-55.

Menne, J., J. K. Park, M. Boehne, M. Elger, C. Lindschau, T. Kirsch, M. Meier, F. Gueler, A. Fiebeler, F. H. Bahlmann, M. Leitges, and H. Haller, 2004, Diminished loss of proteoglycans and lack of albuminuria in protein kinase C-alpha-deficient diabetic mice: Diabetes, v. 53, p. 2101-9.

Messina, A., D. J. Davies, P. C. Dillane, and G. B. Ryan, 1987, Glomerular epithelial abnormalities associated with the onset of proteinuria in aminonucleoside nephrosis.: Am J Pathol, v. 126, p. 220-229.

Meyer, T. W., P. H. Bennett, and R. G. Nelson, 1999, Podocyte number predicts long-term urinary albumin excretion in Pima Indians with Type II diabetes and microalbuminuria: Diabetologia., v. 42, p. 1341-4.

Mifsud, S. A., T. J. Allen, J. F. Bertram, U. L. Hulthen, D. J. Kelly, M. E. Cooper, J. L. Wilkinson-Berka, and R. E. Gilbert, 2001, Podocyte foot process broadening in experimental diabetic nephropathy: amelioration with renin-angiotensin blockade: Diabetologia., v. 44, p. 878-82.

Milionis, H. J., M. S. Elisaf, A. Tselepis, E. Bairaktari, S. A. Karabina, and K. C. Siamopoulos, 1999, Apolipoprotein(a) phenotypes and lipoprotein(a) concentrations in patients with renal failure: American Journal of Kidney Diseases, v. 33, p. 1100-6.

Miner, J. H., 1999, Renal basement membrane components: Kidney Int, v. 56, p. 2016-2024.

Mogensen, C. E., 1971, Urinary albumin excretion in early and long-term juvenile diabetes: Scandinavian Journal of Clinical & Laboratory Investigation, v. 28, p. 183-93.

Mogensen, C. E., W. F. Keane, P. H. Bennett, G. Jerums, H. H. Parving, P. Passa, M. W. Steffes, G. E. Striker, and G. C. Viberti, 1995, Prevention of diabetic renal disease with special reference to microalbuminuria.[see comment]: Lancet, v. 346, p. 1080-4.

Montine, T. J., D. Y. Huang, W. M. Valentine, V. Amarnath, A. Saunders, K. H. Weisgraber, D. G. Graham, and W. J. Strittmatter, 1996, Crosslinking of apolipoprotein E by products of lipid peroxidation: Journal of Neuropathology & Experimental Neurology, v. 55, p. 202-10.

Muhlhauser, I., P. Sawicki, and M. Berger, 1986, Cigarette-smoking as a risk factor for macroproteinuria and proliferative retinopathy in type 1 (insulin-dependent) diabetes: Diabetologia, v. 29, p. 500-2.

Nakamura, T., C. Ushiyama, S. Osada, M. Hara, N. Shimada, and H. Koide, 2001, Pioglitazone reduces urinary podocyte excretion in type 2 diabetes patients with microalbuminuria: Metabolism: Clinical & Experimental, v. 50, p. 1193-6.

Nakamura, T., C. Ushiyama, N. Shimada, K. Sekizuka, I. Ebihara, M. Hara, and H. Koide, 2000a, Effect of the antiplatelet drug dilazep dihydrochloride on urinary podocytes in patients in the early stage of diabetic nephropathy: Diabetes Care, v. 23, p. 1168-71.

Nakamura, T., C. Ushiyama, S. Suzuki, M. Hara, N. Shimada, I. Ebihara, and H. Koide, 2000b, Urinary excretion of podocytes in patients with diabetic nephropathy: Nephrology Dialysis Transplantation, v. 15, p. 1379-83.

Nakamura, T., C. Ushiyama, S. Suzuki, M. Hara, N. Shimada, K. Sekizuka, I. Ebihara, and H. Koide, 2000c, Urinary podocytes for the assessment of disease activity in lupus nephritis: American Journal of the Medical Sciences, v. 320, p. 112-6.

Neale, T. J., P. P. Ojha, M. Exner, H. Poczewski, B. Ruger, J. L. Witztum, P. Davis, and D. Kerjaschki, 1994, Proteinuria in passive Heymann nephritis is associated with lipid peroxidation and formation of adducts on type IV collagen: J Clin Invest, v. 94, p. 1577-84.

Neale, T. J., R. Ullrich, P. Ojha, H. Poczewski, A. J. Verhoeven, and D. Kerjaschki, 1993, Reactive oxygen species and neutrophil respiratory burst cytochrome b558 are produced by kidney glomerular cells in passive Heymann nephritis: Proc Natl Acad Sci U S A, v. 90, p. 3645-9.

Newman, B., J. V. Selby, M. C. King, C. Slemenda, R. Fabsitz, and G. D. Friedman, 1987, Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins: Diabetologia, v. 30, p. 763-8.

Nickenig, G., O. Jung, K. Strehlow, O. Zolk, W. Linz, B. A. Scholkens, and M. Bohm, 1997, Hypercholesterolemia is associated with enhanced angiotensin AT1-receptor expression: American Journal of Physiology, v. 272, p. H2701-7.

Nishibori, Y., L. Liu, M. Hosoyamada, H. Endou, A. Kudo, H. Takenaka, E. Higashihara, F. Bessho, S. Takahashi, D. Kershaw, V. Ruotsalainen, K. Tryggvason, J. Khoshnoodi, and K. Yan, 2004, Disease-causing missense mutations in NPHS2 gene alter normal nephrin trafficking to the plasma membrane: Kidney International, v. 66, p. 1755-65.

Nishikawa, T., D. Edelstein, X. L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M. A. Yorek, D. Beebe, P. J. Oates, H. P. Hammes, I. Giardino, and M. Brownlee, 2000, Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage: Nature, v. 404, p. 787-90.

Norio, R., L. Hjelt, and N. Hallman, 1964, CONGENITAL NEPHROTIC SYNDROME--AN INHERITED DISEASE?A PRELIMINARY REPORT: Annales Paediatriae Fenniae., v. 10, p. 223-7.

Nosadini, R., M. R. Cipollina, A. Solini, M. Sambataro, A. Morocutti, A. Doria, P. Fioretto, E. Brocco, B. Muollo, and F. Frigato, 1992, Close relationship between microalbuminuria and insulin resistance in essential hypertension and non-insulin dependent diabetes mellitus: Journal of the American Society of Nephrology., v. 3, p. S56-63.

Nosaka, K., T. Takahashi, T. Nishi, H. Imaki, T. Suzuki, K. Suzuki, K. Kurokawa, and H. Endou, 1997, An adenosine deaminase inhibitor prevents puromycin aminonucleoside nephrotoxicity: Free Radical Biology & Medicine, v. 22, p. 597-605.

O'Callaghan, C. A., and B. M. Brenner, 2000, The Kidney at a Glance, Blackwell Science.

O'Donnell, M. P., B. L. Kasiske, and W. F. Keane, 1988, Glomerular hemodynamic and structural alterations in experimental diabetes mellitus: FASEB Journal, v. 2, p. 2339-47.

Ohno, S., K. Hora, T. Furukawa, and H. Oguchi, 1992, Ultrastructural study of the glomerular slit diaphragm in fresh unfixed kidneys by a quick-freezing method: Virchows Archiv. B. Cell Pathology, v. 61, p. 351-8.

Orikasa, M., K. Matsui, T. Oite, and F. Shimizu, 1988, Massive proteinuria induced in rats by a single intravenous injection of a monoclonal antibody: Journal of Immunology., v. 141, p. 807-14.

Osicka, T. M., C. A. Houlihan, J. G. Chan, G. Jerums, and W. D. Comper, 2000a, Albuminuria in patients with type 1 diabetes is directly linked to changes in the lysosome-mediated degradation of albumin during renal passage: Diabetes., v. 49, p. 1579-84.

Osicka, T. M., Y. Yu, S. Panagiotopoulos, S. P. Clavant, Z. Kiriazis, R. N. Pike, L. M. Pratt, L. M. Russo, B. E. Kemp, W. D. Comper, and G. Jerums, 2000b, Prevention of albuminuria by aminoguanidine or ramipril in streptozotocin-induced diabetic rats is associated with the normalization of glomerular protein kinase C: Diabetes, v. 49, p. 87-93.

Osterby, R., H. J. Gundersen, A. Horlyck, J. P. Kroustrup, G. Nyberg, and G. Westberg, 1983, Diabetic glomerulopathy. Structural characteristics of the early and advanced stages: Diabetes, v. 2, p. 79-82.

Otey, C. A., G. B. Vasquez, K. Burridge, and B. W. Erickson, 1993, Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain: Journal of Biological Chemistry, v. 268, p. 21193-7.

Pagtalunan, M. E., P. L. Miller, S. Jumping-Eagle, R. G. Nelson, B. D. Myers, H. G. Rennke, N. S. Coplon, L. Sun, and T. W. Meyer, 1997, Podocyte loss and progressive glomerular injury in type II diabetes: Journal of Clinical Investigation, v. 99, p. 342-8.

Palmen, T., H. Ahola, J. Palgi, P. Aaltonen, P. Luimula, S. Wang, I. Jaakkola, M. Knip, T. Otonkoski, and H. Holthofer, 2001, Nephrin is expressed in the pancreatic beta cells: Diabetologia., v. 44, p. 1274-80.

Palmen, T., S. Lehtonen, A. Ora, D. Kerjaschki, C. Antignac, E. Lehtonen, and H. Holthöfer, 2002, Interaction of Endogenous Nephrin and CD2-Associated Protein in Mouse Epithelial M-1 Cell Line: J Am Soc Nephrol, v. 13, p. 1766-1772.

Parving, H. H., H. Lehnert, J. Brochner-Mortensen, R. Gomis, S. Andersen, P. Arner, D. Irbesartan in Patients with Type, and G. Microalbuminuria Study, 2001, The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes.[see comment]: New England Journal of Medicine., v. 345, p. 870-8.

Patrakka, J., P. Martin, R. Salonen, M. Kestila, V. Ruotsalainen, M. Mannikko, M. Ryynanen, J. Rapola, C. Holmberg, K. Tryggvason, and H. Jalanko, 2002a, Proteinuria and prenatal diagnosis of congenital nephrosis in fetal carriers of nephrin gene mutations: Lancet, v. 359, p. 1575-7.

Patrakka, J., V. Ruotsalainen, P. Reponen, E. Qvist, J. Laine, C. Holmberg, K. Tryggvason, and H. Jalanko, 2002b, Recurrence of nephrotic syndrome in kidney grafts of patients with congenital nephrotic syndrome of the Finnish type: role of nephrin.[see comment]: Transplantation, v. 73, p. 394-403.

Pavenstadt, H., W. Kriz, and M. Kretzler, 2003, Cell biology of the glomerular podocyte: Physiological Reviews., v. 83, p. 253-307.

Pearson, E. R., G. Velho, P. Clark, A. Stride, M. Shepherd, T. M. Frayling, M. P. Bulman, S. Ellard, P. Froguel, and A. T. Hattersley, 2001, Beta-cell genes and diabetes: quantitative and qualitative differences in the pathophysiology of hepatic nuclear factor-1alpha and glucokinase mutations: Diabetes, v. 50, p. S101-7.

Pedrini, M. T., A. S. Levey, J. Lau, T. C. Chalmers, and P. H. Wang, 1996, The effect of dietary protein restriction on the progression of diabetic and nondiabetic renal diseases: a meta-analysis.[see comment]: Annals of Internal Medicine, v. 124, p. 627-32.

Petermann, A. T., R. Krofft, M. Blonski, K. Hiromura, M. Vaughn, R. Pichler, S. Griffin, T. Wada, J. Pippin, R. Durvasula, and S. J. Shankland, 2003, Podocytes that detach in experimental membranous nephropathy are viable: Kidney International, v. 64, p. 1222-31.

Pfeilschifter, J., D. Kunz, and H. Muhl, 1993, Nitric oxide: an inflammatory mediator of glomerular mesangial cells: Nephron, v. 64, p. 518-25.

Pierce, G. B., and P. K. Nakane, 1969, Basement membranes. Synthesis and deposition in response to cellular injury: Lab Invest, v. 21, p. 27-41.

Pisitkun, T., R. F. Shen, and M. A. Knepper, 2004, Identification and proteomic profiling of exosomes in human urine: Proceedings of the National Academy of Sciences of the United States of America, v. 101, p. 13368-73.

Plump, A. S., J. D. Smith, T. Hayek, K. Aalto-Setala, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow, 1992, Severe hypercholesterolemia and atherosclerosis in apolipoprotein E- deficient mice created by homologous recombination in ES cells: Cell, v. 71, p. 343-53.

Pomeranz, A., B. Wolach, J. Bernheim, and Z. Korzets, 1995, Successful treatment of Finnish congenital nephrotic syndrome with captopril and indomethacin: Journal of Pediatrics, v. 126, p. 140-2.

Putaala, H., R. Soininen, P. Kilpelainen, J. Wartiovaara, and K. Tryggvason, 2001, The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death: Human Molecular Genetics, v. 10, p. 1-8.

Raats, C. J., J. van den Born, M. A. Bakker, B. Oppers-Walgreen, B. J. Pisa, H. B. Dijkman, K. J. Assmann, and J. H. Berden, 2000, Expression of agrin, dystroglycan, and utrophin in normal renal tissue and in experimental glomerulopathies: American Journal of Pathology, v. 156, p. 1749-65.

Radice, G. L., M. C. Ferreira-Cornwell, S. D. Robinson, H. Rayburn, L. A. Chodosh, M. Takeichi, and R. O. Hynes, 1997, Precocious mammary gland development in P-cadherin-deficient mice: Journal of Cell Biology, v. 139, p. 1025-32.

Rantanen, M., T. Palmen, A. Patari, H. Ahola, S. Lehtonen, E. Astrom, T. Floss, F. Vauti, W. Wurst, P. Ruiz, D. Kerjaschki, and H. Holthofer, 2002, Nephrin TRAP Mice Lack Slit Diaphragms and Show Fibrotic Glomeruli and Cystic Tubu-

lar Lesions: Journal of the American Society of Nephrology, v. 13, p. 1586-94.

Regele, H. M., E. Fillipovic, B. Langer, H. Poczewki, I. Kraxberger, R. E. Bittner, and D. Kerjaschki, 2000, Glomerular expression of dystroglycans is reduced in minimal change nephrosis but not in focal segmental glomerulosclerosis: Journal of the American Society of Nephrology, v. 11, p. 403-12.

Reiser, J., W. Kriz, M. Kretzler, and P. Mundel, 2000, The glomerular slit diaphragm is a modified adherens junction: Journal of the American Society of Nephrology, v. 11, p. 1-8.

Reiser, J., G. von Gersdorff, M. Loos, J. Oh, K. Asanuma, L. Giardino, M. P. Rastaldi, N. Calvaresi, H. Watanabe, K. Schwarz, C. Faul, M. Kretzler, A. Davidson, H. Sugimoto, R. Kalluri, A. H. Sharpe, J. A. Kreidberg, and P. Mundel, 2004, Induction of B7-1 in podocytes is associated with nephrotic syndrome: Journal of Clinical Investigation., v. 113, p. 1390-7.

Reunanen, A., 2004, Suomalainen diabetes: Harvinaisuudesta kansansairaudeksi: Diabetes ja lääkäri, v. 33, p. 6-11.

Ricardo, S. D., J. F. Bertram, and G. B. Ryan, 1994, Reactive oxygen species in puromycin aminonucleoside nephrosis: in vitro studies: Kidney Int, v. 45, p. 1057-69.

Rodewald, R., and M. J. Karnovsky, 1974, Porous substructure of the glomerular slit diaphragm in the rat and mouse: Journal of Cell Biology, v. 60, p. 423-33.

Roselli, S., O. Gribouval, N. Boute, M. Sich, F. Benessy, T. Attié, M.-C. Gubler, and C. Antignac, 2002, Podocin Localizes in the Kidney to the Slit Diaphragm Area: American Journal of Pathology, v. 160, p. 131-139.

Roselli, S., L. Heidet, M. Sich, A. Henger, M. Kretzler, M. C. Gubler, and C. Antignac, 2004, Early glomerular filtration defect and severe renal disease in podocin-deficient mice: Molecular & Cellular Biology, v. 24, p. 550-60.

Ruiz-Ortega, M., O. Lorenzo, M. Ruperez, S. Konig, B. Wittig, and J. Egido, 2000, Angiotensin II activates nuclear transcription factor kappaB through AT(1) and AT(2) in vascular smooth muscle cells: molecular mechanisms.[see comment]: Circulation Research, v. 86, p. 1266-72.

Ruotsalainen, V., P. Ljungberg, J. Wartiovaara, U. Lenkkeri, M. Kestila, H. Jalanko, C. Holmberg, and K. Tryggvason, 1999, Nephrin is specifically located at the slit diaphragm of glomerular podocytes: Proc Natl Acad Sci U S A, v. 96, p. 7962-7.

Ryan, G. B., and M. J. Karnovsky, 1975, An ultrastructural study of the mechanisms of proteinuria in aminonucleoside nephrosis.: Kidney Int, v. 8, p. 219-232.

Saito, T., Y. Ishigaki, S. Oikawa, and T. T. Yamamoto, 2002, Etiologic significance of apolipoprotein E mutations in lipoprotein glomerulopathy: Trend Cardiovasc Med, v. 12, p. 67-70.

Saito, T., S. Oikawa, H. Sato, and J. Sasaki, 1999, Lipoprotein glomerulopathy: Renal lipidosis induced by novel apolipoprotein E variants: Nephron, v. 83, p. 193-201.

Saleem, M. A., L. Ni, I. Witherden, K. Tryggvason, V. Ruotsalainen, P. Mundel, and P. W. Mathieson, 2002, Co-localization of nephrin, podocin, and the actin cytoskeleton : evidence for a role in podocyte foot process formation: American Journal of Pathology, v. 161, p. 1459-66.

Schnabel, E., J. M. Anderson, and M. G. Farquhar, 1990, The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerular epithelium: Journal of Cell Biology, v. 111, p. 1255-63.

Schwarz, K., M. Simons, J. Reiser, M. A. Saleem, C. Faul, W. Kriz, A. S. Shaw, L. B. Holzman, and P. Mundel, 2001, Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin: Journal of Clinical Investigation., v. 108, p. 1621-9.

Seaquist, E. R., F. C. Goetz, S. Rich, and J. Barbosa, 1989, Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy.[see comment]: New England Journal of Medicine, v. 320, p. 1161-5.

Seliger, S. L., C. Davis, and C. Stehman-Breen, 2001, Gender and the progression of renal disease: Current Opinion in Nephrology & Hypertension, v. 10, p. 219-25.

Sellin, L., T. B. Huber, P. Gerke, I. Quack, H. Pavenstadt, and G. Walz, 2003, NEPH1 defines a novel family of podocin interacting proteins: FASEB Journal, v. 17, p. 115-7.

Sharma, M., R. Sharma, A. S. Greene, E. T. McCarthy, and V. J. Savin, 1998, Documentation of angiotensin II receptors in glomerular epithelial cells: American Journal of Physiology, v. 274.

Shih, N. Y., J. Li, R. Cotran, P. Mundel, J. H. Miner, and A. S. Shaw, 2001, CD2AP localizes to the slit diaphragm and

binds to nephrin via a novel C-terminal domain: American Journal of Pathology., v. 159, p. 2303-8.

Shih, N. Y., J. Li, V. Karpitskii, A. Nguyen, M. L. Dustin, O. Kanagawa, J. H. Miner, and A. S. Shaw, 1999, Congenital nephrotic syndrome in mice lacking CD2-associated protein: Science, v. 286, p. 312-5.

Siamopoulos, K. C., M. S. Elisaf, H. T. Bairaktari, M. B. Pappas, G. D. Sferopoulos, and N. G. Nikolakakis, 1995, Lipid parameters including lipoprotein (a) in patients undergoing CAPD and hemodialysis: Peritoneal Dialysis International, v. 15, p. 342-7.

Simons, M., K. Schwarz, W. Kriz, A. Miettinen, J. Reiser, P. Mundel, and H. Holthofer, 2001, Involvement of lipid rafts in nephrin phosphorylation and organization of the glomerular slit diaphragm: American Journal of Pathology, v. 159, p. 1069-77.

Smoyer, W. E., P. Mundel, A. Gupta, and M. J. Welsh, 1997, Podocyte alpha-actinin induction precedes foot process effacement in experimental nephrotic syndrome: American Journal of Physiology, v. 273, p. F150-7.

Solin, M. L., S. Pitkanen, J. W. Taanman, and H. Holthofer, 2000, Mitochondrial dysfunction in congenital nephrotic syndrome: Lab Invest, v. 80, p. 1227-32.

Sprecher, E., R. Bergman, G. Richard, R. Lurie, S. Shalev, D. Petronius, A. Shalata, Y. Anbinder, R. Leibu, I. Perlman, N. Cohen, and R. Szargel, 2001, Hypotrichosis with juvenile macular dystrophy is caused by a mutation in CDH3, encoding P-cadherin: Nature Genetics, v. 29, p. 134-6.

Steffes, M. W., D. Schmidt, R. McCrery, J. M. Basgen, and G. The International Diabetic Nephropathy Study, 2001, Glomerular cell number in normal subjects and in type 1 diabetic patients: Kidney International, v. 59, p. 2104-13.

Striker, G. E., and L. J. Striker, 1985, Glomerular cell culture: Laboratory Investigation, v. 53, p. 122-31.

Suganami, T., M. Mukoyama, K. Mori, H. Yokoi, M. Koshikawa, K. Sawai, S. Hidaka, K. Ebihara, T. Tanaka, A. Sugawara, H. Kawachi, C. Vinson, Y. Ogawa, and K. Nakao, 2005, Prevention and reversal of renal injury by leptin in a new mouse model of diabetic nephropathy: FASEB Journal, v. 19, p. 127-9.

Sugimoto, H., Y. Hamano, D. Charytan, D. Cosgrove, M. Kieran, A. Sudhakar, and R. Kalluri, 2003, Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria: Journal of Biological Chemistry, v. 278, p. 12605-8.

Suzuki, A., T. Ito, E. Imai, M. Yamato, H. Iwatani, H. Kawachi, and M. Hori, 2003, Retinoids Regulate the Repairing Process of the Podocytes in Puromycin Aminonucleoside-induced Nephrotic Rats: Journal of the American Society of Nephrology, v. 14, p. 981-91.

Tarnow, L., P. Rossing, F. S. Nielsen, B. V. Hansen, J. Dyerberg, and H. H. Parving, 1996, Increased plasma apolipoprotein (a) levels in IDDM patients with diabetic nephropathy: Diabetes Care, v. 19, p. 1382-7.

Thakur, V., P. D. Walker, and S. V. Shah, 1988, Evidence suggesting a role for hydroxyl radical in puromycin aminonucleoside-induced proteinuria.: Kidney Int, v. 34, p. 494-499.

Thomas, P. E., B. L. Wharram, M. Goyal, J. E. Wiggins, L. B. Holzman, and R. C. Wiggins, 1994, GLEPP1, a renal glomerular epithelial cell (podocyte) membrane protein-tyrosine phosphatase. Identification, molecular cloning, and characterization in rabbit: Journal of Biological Chemistry, v. 269, p. 19953-62.

Timpl, R., 1989, Structure and biological activity of basement membrane proteins: Eur J Biochem, v. 180, p. 487-502.

Tisch, R., X. D. Yang, S. M. Singer, R. S. Liblau, L. Fugger, and H. O. McDevitt, 1993, Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice.[see comment]: Nature, v. 366, p. 72-5.

Tisher, C. C., and K. M. Madsen, 1991, Anatomy of the kidney, *in* B. M. Brenner, and F. C. Rector, eds., The Kidney: Philadelphia, W.B. Saunders Company, p. 3-75.

Toft, I., K. H. Bonaa, J. Eikrem, A. L. Bendiksen, H. Iversen, and T. Jenssen, 2002, Microalbuminuria in hypertension is not a determinant of insulin resistance: Kidney International., v. 61, p. 1445-52.

Topham, P. S., H. Kawachi, S. A. Haydar, S. Chugh, T. A. Addona, K. B. Charron, L. B. Holzman, M. Shia, F. Shimizu, and D. J. Salant, 1999, Nephritogenic mAb 5-1-6 is directed at the extracellular domain of rat nephrin: Journal of Clinical Investigation, v. 104, p. 1559-66.

Toyoda, M., D. Suzuki, T. Umezono, G. Uehara, M. Maruyama, M. Honma, T. Sakai, and H. Sakai, 2004, Expression

of human nephrin mRNA in diabetic nephropathy: Nephrology Dialysis Transplantation, v. 19, p. 380-5.

Tryggvason, K., 1999, Unraveling the mechanisms of glomerular ultrafiltration: nephrin, a key component of the slit diaphragm: Journal of the American Society of Nephrology, v. 10, p. 2440-5.

Tsukaguchi, H., H. Yager, J. Dawborn, L. Jost, J. Cohlmia, P. F. Abreu, A. B. Pereira, and M. R. Pollak, 2000, A locus for adolescent and adult onset familial focal segmental glomerulosclerosis on chromosome 1q25-31: Journal of the American Society of Nephrology, v. 11, p. 1674-80.

Tuomilehto, J., M. Karvonen, J. Pitkaniemi, E. Virtala, K. Kohtamaki, L. Toivanen, and E. Tuomilehto-Wolf, 1999, Record-high incidence of Type I (insulin-dependent) diabetes mellitus in Finnish children. The Finnish Childhood Type I Diabetes Registry Group.[see comment]: Diabetologia, v. 42, p. 655-60.

Vaarala, O., M. Knip, J. Paronen, A. M. Hamalainen, P. Muona, M. Vaatainen, J. Ilonen, O. Simell, and H. K. Akerblom, 1999, Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes: Diabetes, v. 48, p. 1389-94.

Vauhkonen, I., L. Niskanen, E. Vanninen, S. Kainulainen, M. Uusitupa, and M. Laakso, 1998, Defects in insulin secretion and insulin action in non-insulin-dependent diabetes mellitus are inherited. Metabolic studies on offspring of diabetic probands: Journal of Clinical Investigation., v. 101, p. 86-96.

Verma, R., B. Wharram, I. Kovari, R. Kunkel, D. Nihalani, K. K. Wary, R. C. Wiggins, P. Killen, and L. B. Holzman, 2003, Fyn binds to and phosphorylates the kidney slit diaphragm component nephrin: Journal of Biological Chemistry, v. 278, p. 20716-23.

Vernier, R., B. Papermaster, and R. Good, 1959, Aminonucleoside nephrosis: I. Electronmicroscopic study of renal lesions in rat: J Exp Med, v. 109, p. 115-126.

Viberti, G. C., C. E. Mogensen, P. Passa, R. Bilous, R. Mangili, and S. V. Declaration, 1994, Guidelines for the prevention of diabetic renal failure., *in* C. E. Mogensen, ed., The Kidney and Hypertension in Diabetes mellitus: Boston- Dordrecht- London, Kluwer Academic Publishers, p. 515-527.

Vogelmann, S. U., W. J. Nelson, B. D. Myers, and K. V. Lemley, 2003, Urinary excretion of viable podocytes in health and renal disease: American Journal of Physiology Renal Fluid & Electrolyte Physiology, v. 285.

Wang, S. X., H. Ahola, T. Palmen, M. L. Solin, P. Luimula, and H. Holthofer, 2001a, Recurrence of nephrotic syndrome after transplantation in CNF is due to autoantibodies to nephrin: Experimental Nephrology, v. 9, p. 327-31.

Wang, S. X., P. Mene, and H. Holthofer, 2001b, Nephrin mRNA regulation by protein kinase C: Journal of Nephrology, v. 14, p. 98-103.

Wartiovaara, J., L. G. Ofverstedt, J. Khoshnoodi, J. Zhang, E. Makela, S. Sandin, V. Ruotsalainen, R. H. Cheng, H. Jalanko, U. Skoglund, and K. Tryggvason, 2004, Nephrin strands contribute to a porous slit diaphragm scaffold as: Journal of Clinical Investigation, v. 114, p. 1475-83.

Welsch, T., N. Endlich, W. Kriz, and K. Endlich, 2001, CD2AP and p130Cas localize to different F-actin structures in podocytes: American Journal of Physiology - Renal Fluid & Electrolyte Physiology, v. 281, p. F769-77.

Wen, M., S. Segerer, M. Dantas, P. A. Brown, K. L. Hudkins, T. Goodpaster, E. Kirk, R. C. LeBoeuf, and C. E. Alpers, 2002, Renal injury in apolipoprotein E-deficient mice: Laboratory Investigation, v. 82, p. 999-1006.

Weyer, C., P. A. Tataranni, C. Bogardus, and R. E. Pratley, 2001, Insulin resistance and insulin secretory dysfunction are independent predictors of worsening of glucose tolerance during each stage of type 2 diabetes development: Diabetes Care, v. 24, p. 89-94.

Wharram, B. L., M. Goyal, P. J. Gillespie, J. E. Wiggins, D. B. Kershaw, L. B. Holzman, R. C. Dysko, T. L. Saunders, L. C. Samuelson, and R. C. Wiggins, 2000, Altered podocyte structure in GLEPP1 (Ptpro)-deficient mice associated with hypertension and low glomerular filtration rate: Journal of Clinical Investigation, v. 106, p. 1281-90.

White, K. E., R. W. Bilous, S. M. Marshall, M. El Nahas, G. Remuzzi, G. Piras, S. De Cosmo, and G. Viberti, 2002, Podocyte number in normotensive type 1 diabetic patients with albuminuria: Diabetes, v. 51, p. 3083-9.

Whiteside, C., K. Prutis, R. Cameron, and J. Thompson, 1989, Glomerular epithelial detachment, not reduced charge density, correlates with proteinuria in adriamycin and puromycin nephrosis: Laboratory Investigation, v. 61, p. 650-660.

Yan, K., J. Khoshnoodi, V. Ruotsalainen, and K. Tryggvason, 2002, N-linked glycosylation is critical for the plasma membrane localization of nephrin: Journal of the American Society of Nephrology, v. 13, p. 1385-9.

Yu, Y., C. G. Leng, Y. Kato, and S. Ohno, 1997, Ultrastructural study of glomerular capillary loops at different perfusion pressures as revealed by quick-freezing, freeze-substitution and conventional fixation methods: Nephron, v. 76, p. 452-9.

Yuan, H., E. Takeuchi, and D. J. Salant, 2002a, Podocyte slit-diaphragm protein nephrin is linked to the actin cytoskeleton: American Journal of Physiology - Renal Fluid & Electrolyte Physiology., v. 282, p. F585-91.

Yuan, H., E. Takeuchi, G. A. Taylor, M. McLaughlin, D. Brown, and D. J. Salant, 2002b, Nephrin dissociates from actin, and its expression is reduced in early experimental membranous nephropathy: Journal of the American Society of Nephrology, v. 13, p. 946-56.

Yusuf, S., P. Sleight, J. Pogue, J. Bosch, R. Davies, and G. Dagenais, 2000, Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators.[see comment][erratum appears in 2000 May 4;342(18):1376]: New England Journal of Medicine., v. 342, p. 145-53.

Zanone, M. M., E. Favaro, S. Doublier, B. Lozanoska-Ochser, M. C. Deregibus, J. Greening, G. C. Huang, N. Klein, P. Cavallo Perin, M. Peakman, and G. Camussi, 2005, Expression of nephrin by human pancreatic islet endothelial cells: Diabetologia.

Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda, 1992, Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E: Science, v. 258, p. 468-71.

Zimmet, P., and S. Whitehouse, 1978, Bimodality of fasting and two-hour glucose tolerance distributions in a Micronesian population: Diabetes, v. 27, p. 793-800.