NEPHRIN AND THE PATHOGENESIS OF NEPHROPATHY

EMPHASIS ON TYPE I DIABETES

PETRI AALTONEN

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

ACADEMIC DISSERTATION

To be presented for public discussion, with the permission of the Faculty of Medicine of the University of Helsinki, in the Small Lecture Hall of the Haartman Institute, Haartmaninkatu 3, Helsinki, on May 19th 2006, at 12 o'clock noon.

HELSINKI 2006

SUPERVISED BY

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

REVIEWED BY

Ilkka Pörsti, M.D., Ph.D.
Professor
Department of Internal Medicine
University of Tampere
Finland

and

Tiinamaija Tuomi, M.D., Ph.D.
Docent
Department of Medicine
Helsinki University Central Hospital
Finland

OFFICIAL OPPONENT

Olli Simell, M.D., Ph.D.
Professor
Department of Paediatrics
University of Turku
Finland

ISBN 952-92-0304-7 (paperback)
ISBN 952-10-3110-7 (PDF)
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2006

To Gisele

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals:

I Luimula P, Aaltonen P, Ahola H, Palmén T, Holthöfer H. Alternatively spliced nephrin in experimental disease of the rat. *Pediatric Research* vol. 48, p. 759-62, 2000.

II Aaltonen P, Luimula P, Åström E, Palmén T, Grönholm T, Palojoki E, Jaakkola I, Ahola H, Tikkanen I, Holthöfer H. Changes in the expression of nephrin gene and protein in experimental diabetic nephropathy. *Laboratory Investigation* vol. 81, p. 1185-90, 2001.*

III Kelly DJ, Aaltonen P, Cox AJ, Rumble JR, Langham R, Panagiotopoulos S, Jerums G, Holthöfer H, Gilbert RE. Expression of the slit-diaphragm protein, nephrin, in experimental diabetic nephropathy: differing effects of anti-proteinuric therapies. *Nephrology Dialysis Transplantation* vol. 17, p. 1327-32, 2002.

IV Aaltonen P, Rinta-Valkama J, Pätäri A, Tossavainen P, Palmén T, Kulmala P, Knip M, Holthöfer H. Circulating antibodies to nephrin in patients with type 1 diabetes. Submitted.

Publication I has been used as a part of the thesis of Ph.D. Pauliina Luimula.

* An erratum regarding the details of experimental animal group time point designations has been sent for publication to the *Laboratory Investigation*. Corrected time point designations are used throughout this thesis, and the erratum is available in the Original Publications section.

ABBREVIATIONS

ACE	Angiotensin converting enzyme
AER	Albumin excretion rate
AGEs	Advanced glycation end products
AKT	A member of the serine/threonine-protein kinase family
ANOVA	Analysis of variance, a statistical test
Arp2/3	Actin related protein 2/3 complex
ATP	Adenoside triphosphate
BB	Biobreeding rat model of type 1 diabetes
CASK	Calcium/calmodulin-dependent serine protein kinase
CD2AP	CD2-associated protein
cDNA	Complementary deoxyribonucleic acid
CNF	Congenital nephrotic syndrome of the Finnish type
cpm	Counts per minute, a scintillation measure unit of radioactivity
CTGF	Connective tissue growth factor
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate, a generic term referring to the four deoxyribonucleotides
DTT	Dithiothreitol, a chemical reagent
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ERK/MAPK	Extracellular signal-regulated kinase = mitogen-activated protein kinase
ESRD	End-stage renal disease
FAT/Fat1	Drosophila fat tumor suppressor protein homologue of the cadherin superfamily, see also MEGF1/Fat2
GAD	Glutamic acid decarboxylase
GADA	Glutamic acid decarboxylase autoantibodies
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GLEPP1	Glomerular epithelial protein 1
GLUT1,-2,-4	Glucose transporter proteins
HLA	Human leukocyte antigen
IA	Insulin antibodies (antibodies to exogenous insulin), see also IAA
IA-2	Islet antigen 2
IA-2A	Islet antigen 2 autoantibodies
IAA	Insulin autoantibodies, see also IA
ICA	Islet cell autoantibodies
ICAM-1	Intercellular adhesion molecule 1 (CD54)
IDDM	Insulin-dependent diabetes mellitus, type 1 diabetes
INS-VNTR	Insulin gene variable number of tandem repeats
JAM4	Junctional adhesion molecule 4
kDa	Kilodalton, a molecular weight designation
KDP	Komeda diabetes prone rat
LADA	Latent autoimmune diabetes in adults
LAP	A family of proteins containing **l**eucine-rich repeats **a**nd a **P**DZ-domain, see also PDZ
LDL	Low density lipoprotein
LETL	Long-Evans Tokushima lean rat
LRP	**L**ow density lipoprotein **r**eceptor -related **p**rotein family

MAGI1,-2	Membrane-associated guanylate-kinase inverted proteins
MAPK/ERK	Mitogen-activated protein kinase = extracellular signal-regulated kinase
MEGF1/Fat2	Protein 1 with multiple EGF-like domains, closely similar to the Fat1-protein
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MODY	Maturity-onset diabetes of the young
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
Neph1,-2,-3	Nephrin related genes also known as KIRREL,-2,-3, FILTRIN or NLG
NF-κB	Nuclear factor kappa B, a latent primary transcription factor found in all cell types
NHERF-2	Na^+/H^+ -exchanger regulatory factor 2
NIDDM	Non-insulin dependet diabetes mellitus, type 2 diabetes
NOD	Non-obese diabetic mice
NPHS1	Nephrosis 1, the gene encoding nephrin
NPHS2	Nephrosis 2, the gene encoding podocin
OMIM	Online Mendelian Inheritance in Man, an online catalog of human genes and genetic disorders
PAI-1	Plasminogen activator inhibitor 1
PAN	Puromycin aminonucleoside nephrosis model
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF- β	Platelet derived growth factor beta
PDZ	An interaction domain of proteins (first described in proteins **P**ostsynaptic density 95/**D**iscs-large/**Z**onula occludens 1)
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PPAR-γ	Peroxisome proliferative activated receptor gamma
PTK	Protein tyrosine kinase
PTPase	Protein-tyrosine phosphatase
RAS	Renin-angiotensin (-aldosterone) system
RIP-LCMV	Rat insulin promoter - lymphocytic choriomeningitis virus –model of type 1 diabetes
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RU	Relative units
SD	Standard deviation
SEM	Standard error of the mean
STZ	Streptozotocin, a toxic nitrosurea derivative
TBST	Tris-buffered saline with Tween-20
TGF-β	Transforming growth factor beta
TRPC6	Transient receptor potential cation channel, subfamily C, member 6
VASP	Vasodilator-stimulated phosphoprotein
VEGF/VPF	Vascular endothelial growth factor = vascular permeability factor
VNTR	see INS-VNTR
VPF/VEGF	Vascular permeability factor = vascular endothelial growth factor
ZO-1	Zonula occludens 1 protein

ABSTRACT

End-stage renal disease is an increasingly common pathologic condition, with a current incidence of 87 per million inhabitants in Finland. It is the end point of various nephropathies, most common of which is the diabetic nephropathy. This thesis focuses on exploring the role of nephrin in the pathogenesis of diabetic nephropathy. Nephrin is a protein of the glomerular epithelial cell, or podocyte, and it appears to have a crucial function as a component of the filtration slit diaphragm in the kidney glomeruli. Mutations in the nephrin gene *NPHS1* lead to massive proteinuria. Along with the originally described location in the podocyte, nephrin has now been found to be expressed in the brain, testis, placenta and pancreatic beta cells. In type 1 diabetes, the fundamental pathologic event is the autoimmune destruction of the beta cells. Autoantibodies against various beta cell antigens are generated during this process. Due to the location of nephrin in the beta cell, we hypothesized that patients with type 1 diabetes may present with nephrin autoantibodies. We also wanted to test whether such autoantibodies could be involved in the pathogenesis of diabetic nephropathy.

The puromycin aminonucleoside nephrosis model in the rat, the streptozotocin model in the rat, and the non-obese diabetic mice were studied by immunochemical techniques, *in situ* –hybridization and the polymerase chain reaction –based methods to resolve the expression of nephrin mRNA and protein in experimental nephropathies. To test the effect of antiproteinuric therapies, streptozotocin-treated rats were also treated with aminoguanidine or perindopril. To detect nephrin antibodies we developed a radioimmunoprecipitation assay and analyzed follow-up material of 66 patients with type 1 diabetes.

In the puromycin aminonucleoside nephrosis model, the nephrin expression level was uniformly decreased together with the appearance of proteinuria. In the streptozotocin-treated rats and in non-obese diabetic mice, the nephrin mRNA and protein expression levels were seen to increase in the early stages of nephropathy. However, as observed in the streptozotocin rats, in prolonged diabetic nephropathy the expression level decreased. We also found out that treatment with perindopril could not only prevent proteinuria but also a decrease in nephrin expression in streptozotocin-treated rats. Aminoguanidine did not have an effect on nephrin expression, although it could attenuate the proteinuria.

Circulating antibodies to nephrin in patients with type 1 diabetes were found, although there was no correlation with the development of diabetic nephropathy. At diagnosis, 24% of the patients had these antibodies, while at 2, 5 and 10 years of disease duration the respective proportions were 23%, 14% and 18%. During the total follow-up of 16 to 19 years after diagnosis of diabetes, 14 patients had signs of nephropathy and 29% of them tested

positive for nephrin autoantibodies in at least one sample.

In conclusion, this thesis work could show changes of nephrin expression along with the development of proteinuria. The autoantibodies against nephrin are likely generated in the autoimmune process leading to type 1 diabetes. However, according to the present work it is unlikely that these autoantibodies are contributing significantly to the development of diabetic nephropathy.

1 INTRODUCTION

The discovery of nephrin in 1998 (Kestilä M et al., 1998) opened a window on our understanding of the structure and function of the slit diaphragm in greater detail than before. Nephrin was apparently a protein expressed specifically in the podocytes of the kidney glomeruli. Since the absence of nephrin in the congenital nephrotic syndrome of the Finnish type results in massive proteinuria, nephrin was obviously a crucial component of the slit diaphragm. Proteinuria is a hallmark of nephropathies, including diabetic nephropathy. The present study was motivated by the need to enhance our understanding on the basic underlying pathological processes of proteinuria involving the podocyte. To begin exploring the nephrin behaviour in experimental proteinuria, we chose to study the puromycin aminonucleoside nephrosis model in the rat which resembles the human minimal change nephropathy and presents with transient, massive proteinuria. Thereafter the diabetic nephropathy was studied using the streptozotocin model in the rat and non-obese diabetic mice.

Nephrin was soon shown to be localized in the pancreatic beta cells (Palmen T et al., 2001). While little is still known of the specific functions of nephrin in the beta cells, a variety of beta cell epitopes are known to act as autoantigens in diabetes (Palmer JP et al., 1983; Baekkeskov S et al., 1990; Lan MS et al., 1996). This new information with the interesting report that experimentally administered nephrin antibodies cause a massive proteinuria (Topham PS et al., 1999) led us to hypothesize that nephrin may also act as an autoantigen in type 1 diabetes. To resolve this issue, we developed an appropriate radioimmunoprecipitation assay and analyzed well-characterized follow-up material of patients with type 1 diabetes. Furthermore, we wanted to find out whether the presence of nephrin autoantibodies could influence the development of diabetic nephropathy.

2 REVIEW OF THE LITERATURE

2.1 Structure and function of the kidney

2.1.1 Kidney in overview

In the kidneys (Figure 2.1.1A), blood is filtered through the capillaries in the glomerulus to form a primary filtrate resembling blood plasma. This filtrate then passes through a tubular system, in which the processes of tubular reabsorption and tubular secretion take place. The resulting final urine is a concentrate of waste metabolites, whilst water, important proteins or metabolites such as glucose and necessary electrolytes are efficiently conserved. Normal urine contains only minute amounts of protein, mainly shed or secreted from tubular cells (Ganong WF, 2003b).

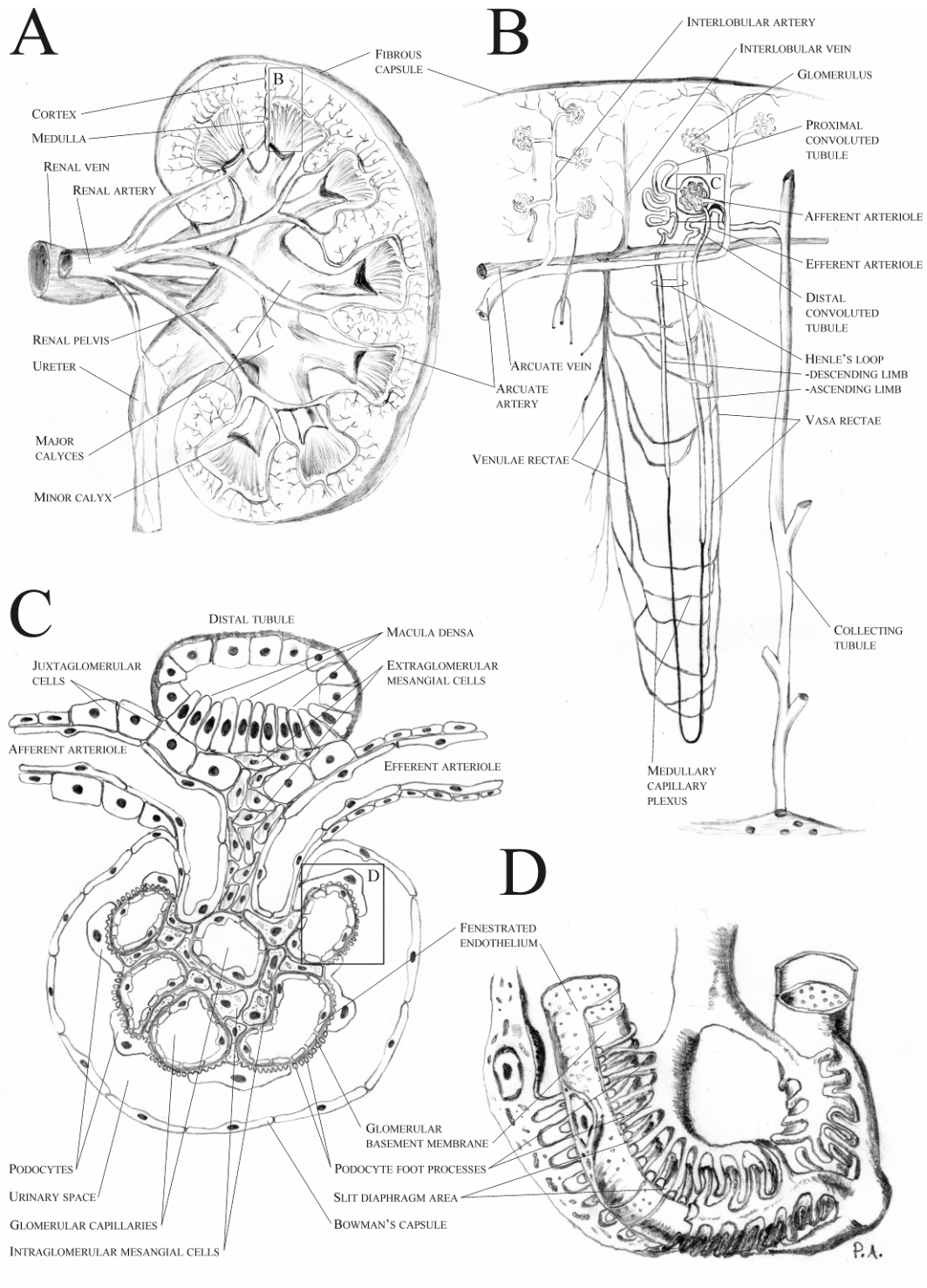
2.1.2 The nephron

The functional unit in the kidney is the nephron (Figure 2.1.1B). There are approximately 0.7 million nephrons in each human kidney, although great individual variation, associated mainly with birthweight, occurs (Hughson M et al., 2003). Each nephron consists of a tuft of capillary loops, or the glomerulus, and a tubular system, as illustrated in Figure 2.1.1B. Glomerulus is a structure of thin capillary loops in the kidney cortex. The glomeruli are responsible for the primary filtration of plasma in the process of urine formation. The tubular system allows the reabsorption of water and electrolytes, which mainly takes place in the loop of Henle. There is also a hormonally regulated mechanism to adjust the release of water into urine in the distal part of the tubular system and collecting duct. The blood flow to the glomerulus is supplied by the afferent arteriole, while an efferent arteriole drains the glomerular capillaries. The efferent arteriole then divides again into multiple capillaries to form a capillary network, which surrounds the tubules. Some of these capillaries, called the vasa recta, also dip to the medulla of the kidney alongside with the loops of Henle. This medullary capillary plexus and the specialized endothelium in these vessels facilitate the reabsorption of water from the tubules to the capillary network (Ganong WF, 2003b).

2.1.3 Nephrogenesis

Nephrogenesis begins at a phase in the development where two tissues of different embryologic origins exist: the metanephric mesenchymal blastema and the

Figure 2.1.1. This illustration displays the anatomy of a coronally cut left kidney (A), the nephron and its associated vasculature (B), a schematic representation of the glomerulus (C), and a view of podocyte foot processes surrounding a glomerular capillary (D). Drawn after Ganong WF (2003b), Gartner LP et al. (1997a) and Netter FH (1997).


A
B
Fibrous capsule
Cortex
Medulla
Renal vein
Renal artery
Renal pelvis
Ureter
Major calyces
Minor calyx
Arcuate artery
B
Interlobular artery
Interlobular vein
Glomerulus
Proximal convoluted tubule
C
Afferent arteriole
Efferent arteriole
Distal convoluted tubule
Arcuate vein
Henle's loop
-descending limb
-ascending limb
Vasa rectae
Venulae rectae
Collecting tubule
Medullary capillary plexus
C
Distal tubule
Macula densa
Juxtaglomerular cells
Extraglomerular mesangial cells
Afferent arteriole
Efferent arteriole
D
Podocytes
Urinary space
Glomerular capillaries
Intraglomerular mesangial cells
Glomerular basement membrane
Podocyte foot processes
Slit diaphragm area
Bowman's capsule
D
Fenestrated endothelium
P.A.

ureteric bud arising from the Wolffian duct. The ureteric bud is induced by the mesenchymal cells to grow and branch into the blastema at weeks 8-9 in human embryonal development. As depicted in Figure 2.1.2, in a series of interactions between these two tissues, the mesenchymal cells condense to the tip of the branching ureteric bud (A-C), convert into epithelial cells (D), form a comma-shaped body (E), an S-shaped body (F), and then differentiate into specialized epithelial cell types. Subsequently, capillary vessels grow into the lower cleft of the S-shaped body (G) and the glomerulus forms (H). The distal part of the S-shaped body is also elongated to form the tubular part of the nephron. Finally, the cells of the mesenchymal blastema have formed the epithelium from the glomerulus until the distal tubulus, and the ureteric bud has formed the collecting duct system and its branches (Horster M et al., 1997).

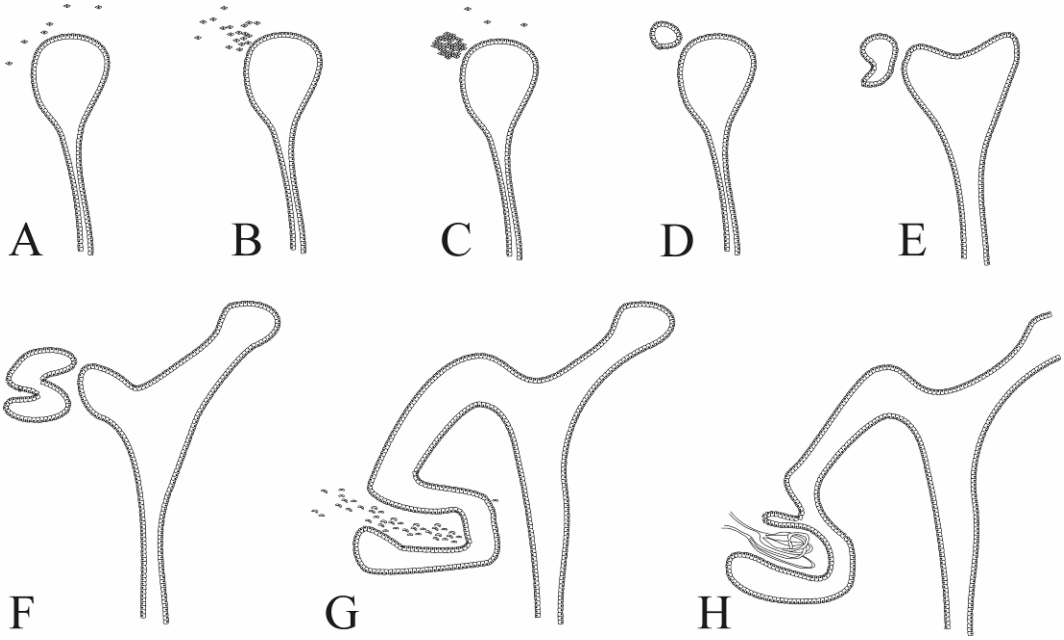


Figure 2.1.2. Development of the nephron from the ureteric bud to the formation of the glomerulus. See text for further details. Modified from Horster M et al., 1997.

2.1.4 Glomerulus

Glomerulus is a tight tuft of capillary loops, surrounded by the visceral glomerular epithelial cells, or podocytes, and their foot processes. This structure is surrounded by the Bowman's capsule made up of the parietal glomerular epithelial cells, which direct the primary urine to the tubular system. A more detailed view of the glomerular filtration barrier shows that there is a finely composed structure responsible for the primary filtration of plasma (Figures 2.1.1C and D). Between the blood flowing in the capillaries of a glomerulus and the urinary space are a fenestrated endothelium,

a specialized glomerular basement membrane (GBM) and the glomerular visceral epithelial cell layer, the podocytes (Gartner LP et al., 1997a). Mesangial cells are located between the capillary loops. They keep the capillary tuft together and secrete various substances essential to the function of the glomerular filter, e.g. components of the basement membrane, addressed later on in this review in chapter 2.5. The filtration function of the kidney is often measured by the glomerular filtration rate (GFR), which is defined as the volume of fluid filtered from the blood in renal glomerular capillaries into the urinary space in Bowman's capsule per unit of time. Mesangial cells also have contractile properties to regulate the GFR by altering the tension around the glomerular capillaries (Stockand JD et al., 1998). The fenestrated capillary endothelium has pores with a diameter of 70-90 nm and allows even fairly large proteins to reach the underlying basement membrane. The pores are rimmed with anionic glycoproteins and lipids (Hjalmarsson C et al., 2004). However, not all of the molecules reaching the 300 nm thick basement membrane are allowed to pass through due to its anionic charge and dense structure (Ganong WF, 2003b). The main constituents of the GBM are type IV collagen and laminin, while large-sized heparan sulphate proteoglycans such as agrin and perlecan as well as fibronectin and nidogen are also components of this structure (Miner JH, 2003). The podocytes and their interdigitating foot processes surround the loops of capillary vessels from all exposed aspects. Between the foot processes is left a thin space of approximately 25 nm bridged by a slit-like membrane called the filtration slit diaphragm (Figure 2.1.1D). The forming filtrate must also pass the filtration slits, which further hinders the passage of larger proteins. Alternatively, proteins may also be passed actively through the cells in the filtration barrier in a process called transcytosis (Tischer CC et al., 1991). Ultimately, the filtration system as a whole effectively excludes the passage of substances larger than 8 nm in diameter. However, negatively charged molecules are repelled by the rims of the endothelial pores and the basement membrane so that filtration of anionic substances of 4 nm in diameter is only half of neutral substances of comparable size (Ganong WF, 2003b).

In recent years, several structural-functional proteins of the filtration slit membrane have been discovered. The best known structural protein is nephrin. The gene encoding nephrin, *NPHS1*, was found in 1998 (Kestilä M et al., 1998). This discovery was the result of longstanding research on the genetic background of the congenital nephrotic syndrome of the Finnish type (CNF), a condition leading to massive proteinuria already *in utero*. In CNF, mutations of the gene *NPHS1* lead to truncation of protein synthesis and absence of functional nephrin (Lenkkeri U et al., 1999). This was a demonstration of the crucial role of the filtration slit in maintaining the function of the glomerular filtration barrier. Intensive research on other podocyte proteins contributing to the integrity of the filtration slit membrane ensued. Presently, such known proteins associated to nephrin and filtration slit are the Neph-proteins, podocin, CD2-associated protein (CD2AP), FAT, densin, and P-cadherin. Also podocalyxin and various other proteins are important in the podocyte function. These proteins and their

interactions are discussed further in chapter 2.6.

2.1.5 Tubulus

The tubular system consists of the proximal tubule, the loop of Henle, and the distal tubule (Figure 2.1.1.B). The proximal tubule begins from the Bowman's capsule surrounding the glomerulus. As the proximal tubule is highly convoluted near the glomerulus this most proximal segment is known as the pars convoluta. The more distal portion of the proximal tubule is straighter, known as the pars recta. The pars recta may also be called the descending thick limb of Henle's loop. It descends towards the medulla to become continuous with the thin limb of Henle's loop. About 80% of sodium, chloride, and water, as well as main part of the glucose, free amino acids and protein in the glomerular primary filtrate are normally resorbed by the cuboidal epithelial cells of the proximal tubule (Gartner LP et al., 1997a).

The thin limb of Henle's loop is composed of squamous epithelial cells, and its descending part is highly permeable to water, as well as to urea, sodium, chloride and other ions. The ascending part of the thin limb is only moderately permeable to water. This difference in water permeability is important in the reabsorption of water into the capillary vessels (Gartner LP et al., 1997a). However, the greater details of the countercurrent multiplier and exchange mechanisms are beyond the scope of this review, but are found in the review by Ganong WF, 2003b.

The distal tubule begins with pars recta, also known as the ascending thick limb of Henle's loop, and continues as the distal convoluted tubule (pars convoluta). The distal tubule is impermeable to water and urea, so that the urea concentration in the tubular lumen remains high. The cells of pars convoluta have Na^+-K^+ ATPase pumps, which in response to the hormone aldosterone can actively resorb all of the remaining sodium from the lumen of the tubule (Gartner LP et al., 1997a).

The tubular system and the interstitial kidney tissue are typically damaged in conditions with chronic proteinuria and glomerular scarring. Because the tubules are perfused by the capillary vessels arising from glomerular efferent arterioles (Figure 2.1.1.B), advanced glomerulosclerosis leads to tubular ischemia and interstitial fibrosis. Thus, nephrosclerosis involving the entire organ may be the result of long-standing diabetes (Kumar V et al., 1992).

2.2 The endocrine pancreas – structural and functional review

2.2.1 Overview of the pancreas

The pancreas has exocrine and endocrine functions. The largest portion of the pancreatic tissue consists of the exocrine acinar cells, which occupy about 80% of the total volume of the gland. These cells excrete various digestive enzymes via the pancreatic duct into the duodenum, including amylase, lipase, ribonuclease, DNase, and the proenzymes trypsinogen, chymotrypsinogen, procarboxypeptidase,

and elastase. Only 2-4% of the pancreatic tissue consists of cells with endocrine function, while mainly ducts and blood vessels take the rest of the space. The endocrine activity is scattered to the 1-2 million islets of Langerhans, which are approximately 75x175 µm sized oval collections of about 3000 cells surrounded by pancreatic acinar cells. The islets are penetrated by a network of capillaries. Each islet of Langerhans contains at least five cell types: α-cells, β-cells, δ-cells, G cells and F cells (Gartner LP et al., 1997b).

2.2.2 Development of the pancreas

The pancreas is initially derived from separate endodermal dorsal and ventral buds arising from the so-called foregut at week four of embryonic development in human. Later these buds form the main ducts of pancreas (Johansson KA et al., 2002). The dorsal bud develops from just below the notochord in the region of the stomach. The ventral pancreas, originally formed adjacent to the liver, rotates to the left and eventually comes into contact with the dorsal bud by the beginning of week six. The ducts inside each part interconnect, elongate and branch. The terminal parts of the ducts form the exocrine acini and cell clumps migrate away from the walls of smaller branches into the surrounding mesenchyme to give rise to the endocrine islets. The precursory islets then expand through proliferation and by merging with other nearby cell clumps. Cells begin to differentiate into specialized islet cell populations after gestational week 10. Angiogenesis begins in the mesenchyme and by week 13 the islets have a fine capillary network. Finally, at week 17 the beta cells begin to excrete insulin. For a review, see Johansson KA et al., 2002.

2.2.3 Beta cells and other islet cells

Beta cells are the most common cell type in the islets, occupying an estimated proportion of 60-80% of the islet cells (Gartner LP et al., 1997b). They produce insulin and accumulate it into cytoplasmic granules to await regulated excretion. Insulin is encoded in the human by a single gene, *INS*, at chromosomal location 11p15.5 (Harper ME et al., 1981). In the rat and mouse insulin is encoded by two genes with essentially identical protein products (Lomedico P et al., 1979; Wentworth BM et al., 1986). In the human, insulin is synthesized first as a preproinsulin at the rough endoplasmic reticulum. Insulin is directed into the endoplasmic reticulum and thus to its regulated exocytic pathway by a signalling polypeptide fragment, which is cotranslationally cleaved off to yield proinsulin (Eskridge EM et al., 1986). At the trans-Golgi network, proinsulin is packed into vesicles coated with the common packaging-molecule clathrin. A segment of the proinsulin known as the C-peptide is then removed by the endoproteases prohormone convertase 1/3 and 2 (Arvan P et al., 2004). The vesicles then mature and lose their clathrin coat and present finally as the intracellular granules containing insulin. As the beta cell adenoside triphosphate (ATP) level increases, most commonly due to increased blood glucose level, the ATP-sensitive K^+ channels on the cell membrane are closed, the beta cell depolarizes and exocytoses its insulin. Insulin then diffuses through the basal lamina to the adjacent fenestrated capillaries and is carried via the bloodstream to the hepatic portal vein and further to the systemic circulation. Interestingly, nephrin has been found in the islets of Langerhans. It has been reported to

be localized on the beta cells (Palmen T et al., 2001) and/or the endothelium of the capillaries in the islets (Zanone MM et al., 2005).

Insulin acts on specific target cells which express insulin receptors. The effects of insulin are mediated by the insulin receptors and their intracellular ligands, the insulin receptor subtrates 1 and 2, which are located in nearly all cells. In the tissues, especially in skeletal muscle, liver and adipose tissue, insulin has the important and immediate function to increase glucose entry into cells. Binding of insulin to its receptors causes a rapid translocation of facilitative glucose transport molecules to the cell membrane, which allow an increased flux of glucose into the cell (Ganong WF, 2003c). Activation of insulin signalling increases amino acid and potassium entry into insulin-sensitive cells. Insulin also reduces gluconeogenesis and glucose release by the liver, stimulates protein synthesis and increases lipogenesis in adipose tissue. With special reference to the kidney it has been recently shown that insulin also has direct effects on the podocyte as well (Coward RJ et al., 2005b). According to the latest communicated results, nephrin seems to be crucial for the function of insulin in the podocyte (Coward RJ et al., 2005a).

The alpha cells of the islets of Langerhans secrete glucagon, a peptide hormone released in response to low blood glucose levels (Gartner LP et al., 1997b). The main target of glucagon is the liver, where it acts on the hepatocytes causing them to rapidly break down their glycogen stores and release glucose to the bloodstream. Delta cells secrete somatostatin, which inhibits the endocrine activity of the alpha and beta cells. Further, somatostatin reduces motility of the gastrointestinal tract and gallbladder. Somatostatin is released after the digestion of a meal in response to increased blood levels of amino acids, glucose or chylomicrons. G cells release and produce gastrin, a hormone stimulating gastric motility, release of HCl and regeneration of gastric wall cells. The F cells secrete pancreatic polypeptide (PP) and are therefore also known as the PP cells. Pancreatic polypeptide inhibits the exocrine secretions of the pancreas (Gartner LP et al., 1997b).

2.3 Type 1 diabetes

2.3.1 Epidemiology

In Finland, the current incidence of type I diabetes is approximately 54 per 100000 in children under 15 years of age (Knip M et al., 2005), which is the highest in the world. The incidence has been increasing constantly at a mean annual rate of 3% (Onkamo P et al., 1999). The lower end of the incidence scale includes China and Venezuela, where the incidence rates are at 0.1 per 100000 (Karvonen M et al., 2000), representing a more than 500-fold variation among the world population. It is also possible that the onset of this disease is now occurring at an earlier age than before (Karvonen M et al., 1999). The increase in the incidence of as well as the new research

on the genetic background of type 1 diabetes suggests that a yet unknown and increasing environmental factor triggers the disease in some of genetically susceptible individuals.

2.3.2 Pathogenesis

Type 1 diabetes is a disease caused by autoimmunity. The central pathologic event is the triggering of immunological destruction of pancreatic beta cells in the islets of Langerhans. Before the onset of clinical symptoms and almost a total lack of insulin, there is a phase of varied duration with decreasing insulin secretion. The respective gradual destruction of beta cells is primarily mediated by the T-cells of the immune system, and T-cell infiltration is histologically visible in the early prediabetic phase of the disease (Bottazzo GF et al., 1985). Inflammation of the pancreatic islets, insulitis, is preceded by the loss of immunological tolerance towards products of beta cells, mainly insulin. The exact mechanism leading to this loss of immunotolerance is not known, although the central role of autoreactive T-cells (Roep BO, 2003) and particularly the regulatory T-cells (Lan RY et al., 2005) is acknowledged. A pathogenetic model proposes that in genetically susceptible individuals a triggering exogenous factor commences the autoimmunity against beta cells, driven by another environmental antigen, and modified by various environmental factors (Knip M et al., 2005).

During the initial stages of the disease process, the humoral part of the immune system is also activated. This leads to development of circulating antibodies to various “self” antigens. The most important autoantibodies are the glutamic acid decarboxylase autoantibodies (GADA) (Baekkeskov S et al., 1990), islet antigen 2 autoantibodies (IA-2A; Lan MS et al., 1996) and insulin autoantibodies (IAA; Palmer JP et al., 1983). The presence of these autoantibodies is a sign of activation of the autoimmune process and their detection is of clinical use in predicting the onset of diabetes in subjects at risk, e.g. siblings of a type 1 diabetic patient.

2.3.3 Genetic and other risk factors

Genetic predisposition is known to be important for the development of type 1 diabetes (OMIM: 222100). In siblings of a type 1 diabetic patient the average risk for developing the disease is 6-7% (Harjutsalo V et al., 2005). In monozygotic twins with identical genetic configuration the concordance for type 1 diabetes is 30-50% (Kyvik KO et al., 1995; Hyttinen V et al., 2003).

The best characterized genetic factor is the human leukocyte antigen (HLA) gene complex located on the short arm of the chromosome 6 at the band p21.3. The genes within the HLA gene complex are known to explain up to 50% of the genetic component of risk for type 1 diabetes (Vyse TJ et al., 1996). The HLA genes are generally divided into three classes: class I genes (A, B, C and others) encode proteins expressed in all nucleated cells and present peptide fragments derived from cytosolically produced proteins (endogenous pathway of antigen presentation) to CD8+ (cytotoxic) T cells; class II genes (DP, DQ, DR and others) encode heterodimeric membrane proteins expressed in specific antigen-presenting cells (e.g. activated T cells, B cells, dendritic cells, macrophages) and they are involved in the presentation of exogenously derived peptides to the CD4+

(helper) T cells; class III genes contain various genes of the complement system and some cytokines (Kelly MA et al., 2003). Earlier serologic identification of HLA antigens in individuals was used, and the antigens were grouped accordingly to various HLA-serotypes e.g. DR1, DR2, DQ1, DQ2, and so on (Schwartz BD, 1987). In the more recent era of genetics, it has become possible to identify much more detailed differences in the genes encoding the HLA antigens. Today, numerous alleles of HLA genes are known among each serologically defined, broad antigen group. A nomenclature committee under the World Health Organization agrees on the naming conventions of these alleles. Briefly, their current naming convention first describes the gene locus followed by an asterisk and a series of 4 to 8 digits corresponding to the serologic allele family (2 digits), and the allele coding variation (2 digits). The last 4 digits and specific letter suffices are sometimes used for separating further variation (Marsh SG et al., 2002).

In particular, the genes DQB1, DQA1 and DRB1 in the HLA class II are determinants of type 1 diabetes risk. Strong linkage disequilibrium, i.e. the inheritance of alleles at distinct loci together more frequently than expected by chance, is present between many genes in the HLA complex, making it difficult to identify the primary susceptibility determinants of type 1 diabetes (Kelly MA et al., 2003). According to current knowledge, susceptibility is associated with two combinations of DQA1 and DQB1 alleles: DQA1*0301-DQB1*0302 (encoding protein HLA-DQ8) and DQA1*0501-DQB1*0201 (encoding protein HLA-DQ2), while other combinations such as DQA1*0102-DQB1*0602 (encoding protein HLA-DQ6.2) carry a protective effect. Also, two allele families of DRB1 associate with type 1 diabetes risk, the DRB1*04 and the DRB1*03, encoding proteins DR4 and DR3, respectively. Linkage disequilibrium between the DRB1*03 and DQA1*0501-DQB1*0201 allele combination forms the predisposing haplotype DR3-DQ2. Another such linkage between the alleles DRB1*04 and DQA1*0301-DQB1*0302 forms the predisposing haplotype DR4-DQ8 (Kelly MA et al., 2003). Accordingly, the highest genetic risk is conferred by the heterozygous DR3-DQ2/DR4-DQ8 genotype (Ilonen J et al., 2002). Up to 90% of patients with type 1 diabetes carry at least one of the above mentioned predisposing haplotypes, while in other patients susceptibility is carried by other HLA haplotypes (Undlien DE et al., 1999).

The research today has proceeded beyond the study of HLA alleles and is focusing on the search for other genetic factors predisposing to type 1 diabetes. More than 20 candidate loci have been proposed but only three of them are reliably characterized and confirmed. Discussed below, these genes are the *INS-VNTR*, the *CTLA-4* and the *PTPN22*.

In 1984, Bell et al. reported a region near the insulin gene at 11p15 with a variable number of tandemly repeated (VNTR) 14-bp oligonucleotides (Bell GI et al., 1984). Furthermore this minisatellite locus upstream of the insulin gene is suggested to influence the messenger ribonucleic acid (mRNA) expression of insulin and insulin-like growth factor 2 genes in the thymus and pancreas (Pugliese A et al., 1997; Vafiadis P et al., 1997), which is shown to affect the T cell self-tolerance (Anjos S et al., 2004). Depending on the type of the VNTR-sequence it could

increase or decrease self-tolerance (Bennett ST et al., 1995), although the exact molecular mechanisms predisposing to type 1 diabetes are not yet clear.

The cytotoxic T-lymphocyte antigen 4 (CTLA-4) is a transmembrane protein of CD4+ T cells normally taking part in the elimination of self-reactive T cells. Multiple studies have confirmed its association with type 1 diabetes (Nistico L et al., 1996; Kavvoura FK et al., 2005). The mechanism proposed to mediate the risk for type 1 diabetes is a single nucleotide polymorphism in the signal peptide of CTLA-4 believed to reduce the expression of this protein on the cell surface of T cells (Abbas AK et al., 2004).

The gene PTPN22 encodes the lymphoid protein tyrosine phosphatase (LYP), which is another T-cell activation suppressor. Originally described in 2004 (Bottini N et al., 2004), the strong association of a single-nucleotide polymorphism in this gene with the risk for type 1 diabetes has already been confirmed by several studies (Onengut-Gumuscu S et al., 2004; Ladner MB et al., 2005; Zheng W et al., 2005).

The known inherited risk factors leave a broad space for environmental factors as well, since not all of the genetically susceptible individuals develop type 1 diabetes. The exact exogenous triggering mechanism of the autoimmune event leading to destruction of insulin-producing beta cells has not been established conclusively. However, it is believed that certain viral agents such as enterovirus infections could act as the triggering factor initiating autoimmunity, while dietary proteins such as cow milk proteins and particularly bovine insulin are speculated to be the driving antigens maintaining the process (Knip M et al., 2005). In conclusion, type 1 diabetes is a complex disease with multiple different gene loci and environmental factors involved in its pathogenesis.

2.3.4 Clinical course and complications

Most of the patients with type 1 diabetes are under 20 years of age, whilst onset takes place later in a third of cases. Symptoms referring to type 1 diabetes are loss of weight, nausea, fatigue, abdominal pain, polydipsia, polyphagia, and polyuria (Williams G et al., 1994). The onset of symptoms may be abrupt and ketoacidosis is common. The only feasible treatment is insulin, which is administered subcutaneously. The majority of patients require multiple injection therapy consisting of a long-acting insulin and short-acting insulin injections at meals. The aim of the therapy is to maintain a physiologic level of blood glucose. To achieve this the glycated haemoglobin level reflecting the mean blood glucose levels of the last 3-4 months is measured regularly. Failure in maintaining good glycemic balance is a risk for developing complications. As a fundamental metabolic disorder, type 1 diabetes has serious complications affecting mainly the vascular system. The complications are generally divided into macro- and microvascular complications. An increased rate of atherosclerosis in the large vessels, foot ulcers possibly leading to amputation, and a high risk for coronary angiopathy are the macrovascular complications, whereas neuropathy, retinopathy and nephropathy are the distinct microvascular complications. Untreated retinopathy may eventually lead to blindness, whilst nephropathy is an important complication of diabetes as well.

Diabetic nephropathy is a pathologic process presenting first as an increasing loss of protein into the urine, while the ultimate weakening of the GFR leads to end-stage renal disease (ESRD) with retention of protein and waste metabolites. Diabetic nephropathy is the most common cause of ESRD in Finland and elsewhere in the world (United Kingdom Renal Registry, 2003; United States Renal Data System, 2003; Finnish Registry for Kidney Diseases, 2005). In Finland, the incidence of ESRD resulting of diabetic nephropathy in 2004 was approximately 33 per million inhabitants, explaining almost 40% of all new cases of ESRD (Finnish Registry for Kidney Diseases, 2005). This is largely due to the increased incidence of type 2 diabetes.

Patients with diabetic nephropathy are treated with angiotensin converting enzyme (ACE) inhibitors, as they seem to have a renoprotective effect independent of the blood pressure lowering effect (Lewis EJ et al., 1993). Maintaining strict glycemic balance and blood pressure control, avoiding smoking, and, after the onset of proteinuria, applying a low-protein diet are other measures aiming to prevent or ameliorate the kidney complication. Dialysis and kidney transplantation remain as the only therapeutic options at end-stage renal disease. Approximately one third of patients with type 1 diabetes develop renal complications during the course of their disease, while some patients avoid nephropathy even where there is poor glycemic balance or other risk factors such as smoking (Rossing P et al., 1995; Rossing P et al., 2002; Hovind P et al., 2004). This suggests an important role for genetic predisposition, which is more comprehensively addressed in chapter 2.5. The incidence of diabetic nephropathy has been reported to decrease after the application of various modern therapeutic interventions (Bojestig M et al., 1994; Hovind P et al., 2003; Nordwall M et al., 2004), although many studies have announced an unchanged incidence (Rossing P et al., 1995; Esmatjes E et al., 2002; Tryggvason G et al., 2005) or inadequacy of modern therapy to prevent the complication (Svensson M et al., 2003). After all, regardless of the underlying pathogenesis, all types of diabetes lead to increased blood glucose concentrations. This is considered to be an important factor in the complications of all types of diabetes, although other individual and environmental factors clearly contribute to the development of diabetic complications.

2.3.5 Other types of diabetes

The most common type of diabetes is the type 2 diabetes. In this form of the disease, insulin secretion defect is not the primary pathogenic mechanism. Instead, the function of insulin on target cells is reduced. Also other types of diabetes exist. One of them is the maturity-onset diabetes of the young (MODY), where very young patients present with symptoms similar to type 2 diabetes. It is inherited in an autosomal dominant pattern (OMIM: 125850), and several associated genetic defects are known. Gestational diabetes is a transient decrease in glucose tolerance during pregnancy, although it involves an increased risk for developing type 2 diabetes later. The latent autoimmune diabetes in adults (LADA) is a type of diabetes with late-onset autoimmune destruction of pancreatic beta cells. Rare mitochondrial defects can also be a cause of diabetes. The common types of diabetes are also intermixing and the

metabolic syndrome behind the development of type 2 diabetes is fairly common in patients with type 1 diabetes as well. Up to 40% of type 1 diabetic patients have metabolic syndrome (Thorn LM et al., 2005). Furthermore, familial clustering of type 1 and type 2 diabetes is clear, with an increased frequency of type 2 diabetes in families with type 1 diabetes (Dahlquist G et al., 1989) and vice versa (Li H et al., 2001).

2.4 Relevant animal models of renal disease

2.4.1 Puromycin aminonucleoside nephrosis

The puromycin aminonucleoside nephrosis (PAN) in the rat is a commonly used animal model for proteinuria. It is induced by a single intravenous injection of puromycin aminonucleoside. It is an antibiotic with antineoplastic properties and it can cause nephrosis. Thus, in the rat the injection leads to proteinuria at around day 3, while the maximal effect is usually seen at day 10 (Ryan GB et al., 1975). After this, the condition is reversed so that normal kidney function is regained at around day 28 (Ryan GB et al., 1975). The morphological and functional changes resemble the human minimal change nephropathy (Vernier RL et al., 1959). Morphological changes in the PAN model include dilation of tubuli, fusion of podocyte foot processes, alteration of the slit diaphragm and even replacement of the slit diaphragm by occluding-type junctions (Caulfield JP et al., 1976). At later stage, a characteristic detachment of podocytes is seen, allowing contact of the GBM with the urinary space (Messina A et al., 1987). The detailed mechanisms in which puromycin aminonucleoside induces nephrosis are not known. However, treatment with puromycin aminonucleoside of cultured podocytes resulted in impaired adhesion of these cells to type IV collagen and decreased expression of α3β1-integrin, the major integrin attaching the podocytes to the GBM (Krishnamurti U et al., 2001). Another *in vitro* study implicates apoptosis via oxidative stress in the detachment of podocytes from culture substrata (Suzuki T et al., 2001).

2.4.2 Animal models of type 1 diabetes

2.4.2.1 Streptozotocin model

Streptozotocin (STZ) is a toxic nitrosurea derivative first isolated from *Streptomyces achromogenes* (Lewis C et al., 1959; Vavra JJ et al., 1959). STZ has powerful alkylating capabilities and it induces multiple deoxyribonucleic acid (DNA) strand breaks in cells by direct methylation of DNA (Bennett RA et al., 1981). In an animal model a single dose of STZ is given and diabetes develops usually in 1-2 days due to destruction of pancreatic beta cells (Rakieten N et al., 1963). STZ has the ability to efficiently destruct pancreatic beta cells because it is transported to the beta cell by the cell-surface glucose transporter GLUT-2. There are several glucose transporter protein types in various tissues, but GLUT-2 is the constitutive transporter protein of the beta cell and it is fairly

specifically expressed in these cells. This leads to the enhanced uptake of STZ and restriction of greatest damage to the beta cells (Schnedl WJ et al., 1994). STZ is generally used in the rat or the mouse, but STZ also functions in humans and has been used in cancer chemotherapy of e.g. malign insulinomas, pancreatic tumors originating from insulin-secreting cells (Murata E et al., 1985). During antineoplastic treatment regimens involving the administration of STZ the patients are monitored carefully, since in humans STZ is known to cause acute renal tubular toxicity (Sadoff L, 1970; Kintzel PE, 2001) and progressive renal failure can be prevented by drug discontinuance (Ries F et al., 1986). Such acute nephrotoxicity is not seen in rats (Rakieten N et al., 1963; Junod A et al., 1967; Kraynak AR et al., 1995). However, in experimental animals, STZ is carcinogenic: even a single diabetogenic administration induces tumors in rat kidney, liver and pancreas (Rakieten N et al., 1968; Bolzan AD et al., 2002). Although STZ is possibly carcinogenic in humans as well these effects require further study (Bolzan AD et al., 2002). A genotoxicologic work studying the extent and persistence of DNA damage caused by STZ in rat kidney reported that a commonly used diabetogenic dose of 60 mg/kg STZ induced extensive DNA damage which required up to 3 weeks to be repaired. The authors concluded that the STZ model should be avoided when the enhancement of tumorigenesis may be an unwanted complication. Further, they suggest that in studies testing for the effects of any drug a delay of 3 to 4 weeks should be given before the start of treatment to allow the greatest possible repair of DNA damage (Kraynak AR et al., 1995).

The STZ model in the rat and the mouse is most useful in areas of research concerning the adverse effects of hyperglycemia. There is also a modification of this model with multiple low-dose injections of STZ which leads to insulinopenia and development of insulitis with immune destruction of the pancreas. However, in the multiple low-dose model diabetes will develop even in the absence of functional T or B cells (Barlow SC et al., 2004). Thus, other animal models are mainly used in the study of the pathogenesis of autoimmune insulitis leading to type 1 diabetes.

2.4.2.2 Non-obese diabetic mouse

Originally described in 1980, the non-obese diabetic (NOD) mouse is a breed of mice with spontaneously developing autoimmune insulitis at 4-5 weeks of age, including infiltration of T cells into islets of Langerhans, ultimately destroying the pancreatic beta cells (Makino S et al., 1980). The diabetic state usually presents between 12 to 30 weeks of age. In contrast to human type 1 diabetes, the incidence of diabetes in typical colonies of NOD mice is approximately 90% in females but only 60% in the males. In addition to diabetes, NOD mice spontaneously develop autoimmune responses in a variety of tissues, including salivary, lacrimal, thyroid, parathyroid, adrenal, testis, large bowel and red blood cells, and they are also susceptible to the experimental induction of a variety of other autoimmune diseases, including experimental autoimmune thyroiditis, colitis-like wasting disease, encephalomyelitis, and manifestations of systemic lupus erythematosus (Aoki CA et al., 2005). Despite the greater gender

difference in incidence, the NOD mouse can be considered to be a close model of human type 1 diabetes and it is widely used in research of the immunological pathogenesis of diabetes, whilst it is applicable to the study of T cell behaviour in autoimmunity in general (Ridgway WM, 2003).

2.4.2.3 Biobreeding rat

The biobreeding (BB) rat is also an inbred model of diabetes. The BB diabetes-prone rats develop autoimmune diabetes with symptoms presenting at 12 weeks after birth (Nakhooda AF et al., 1977). Similar immunological features are seen as in the NOD mice, but the BB diabetes-prone rats characteristically have a clear T cell lymphopenia, which is not present in the human type 1 diabetes or in the NOD mice. With reference to renal complications, the BB rats seem to have an interesting relative resistance to diabetic nephropathy. This is suggested to be due to increased production of nitric oxide or decreased accumulation of advanced glycation end-products (Feld LG et al., 1995), both of which are important mediators in the development of diabetic nephropathy as discussed in chapter 2.5. Nevertheless, the BB rat has been used in a range of studies concerning the development of diabetic nephropathy.

There exists also another strain of the BB rat, the BB diabetes-resistant rat, which does not develop spontaneous diabetes. However, when BB diabetes-resistant rats are infected with the Kilham's rat virus, they develop autoimmune insulitis and diabetes (Guberski DL et al., 1991). The Kilham's rat virus -induced diabetes in the BB diabetes-resistant rat is therefore a useful model for studying the role of viruses in the pathogenesis of autoimmune beta cell destruction.

2.4.2.4 Transgenic and other animal models of diabetes

Other intriguing models of diabetes include the Long-Evans Tokushima lean (LETL) rat and Komeda diabetes-prone (KDP) rat as well as a model with proteins of the lymphocytic choriomeningitis virus expressed under the rat insulin promoter (RIP-LCMV). The LETL rat is another rat model of type 1 diabetes. This rat strain develops a spontaneous disease with lymphocyte infiltration into pancreatic islets and a sudden onset of diabetic symptoms. The incidence of diabetes is only approximately 20%. No lymphopenia or sex difference in incidence or severity is seen (Kawano K et al., 1991). Subsequently, two substrains have been derived from the LETL rat: a non-diabetic strain and a diabetes-prone strain. The latter strain is called the KDP rat and it has an overall 70% incidence of diabetes (Komeda K et al., 1998). RIP-LCMV is an interesting model for autoimmune diabetes developed independently by two groups in 1991 (Ohashi PS et al., 1991; Oldstone MB et al., 1991). These mice lose the tolerance for the LCMV epitopes and develop diabetes after infection with LCMV due to an immune response against the viral epitopes in the beta cells. Various transgenic animals with increased or decreased number of gene copies of diabetes-related genes have recently been engineered. Also the possibility to develop mouse models with tissue-specific gene knock-outs of single genes has been utilized in recent diabetes research. See Table 2.4 for summary of several informative transgenic animal

models of diabetes. There exists also a range of other animals such as hamsters, rabbits, dogs, and apes with spontaneous development of autoimmune diabetes resulting in complications such as diabetic nephropathy (Kramer JW et al., 1980; Conaway HH et al., 1981). However, most of these animals are larger than the rodents described above and are not convenient for laboratory research. Furthermore, ethical principles favour the use of rodents in animal studies.

Table 2.4: Transgenic animal models in diabetes research

Target gene	Model type	Effects	Reference
Insulin receptor	Mouse gene knockout	Neonatal lethality	Joshi RL et al., 1996
Insulin receptor	Expression of kinase deficient human insulin receptor in transgenic mice	Decreased insulin sensitivity *in vivo*	Chang PY et al., 1994
Insulin receptor	Mouse pancreatic beta cell – specific gene knockout	Insulin secretory defect similar to human type 2 diabetes	Kulkarni RN et al., 1999
Insulin receptor substrate -1	Mouse gene knockout	Insulin resistance and growth retardation	Tamemoto H et al., 1994
Insulin receptor substrate -2	Mouse gene knockout	Type 2 diabetes –like symptoms, liver insulin resistance and lack of compensatory beta cell hyperplasia	Withers DJ et al., 1998; Kubota N et al., 2000
Glucokinase	Mouse gene knockout	Perinatal lethality with hyperglycaemia	Grupe A et al., 1995
HLA DRB1 and DQA1/DQB1	Expression of predisposing human DR3 and/or DQ8 in transgenic mice	Development of spontaneous insulitis	Abraham RS et al., 2000
GLUT4 receptor	Expression of normal human GLUT4 receptor in transgenic mice	Efficient glycemic control	Liu ML et al., 1993
PPAR-γ	Mouse pancreatic beta cell – specific gene knockout	Abnormalities in islet mass, normal glucose homeostasis	Rosen ED et al., 2003

2.5 Mechanisms of diabetic nephropathy

2.5.1 Pathological changes in the function and structure of the glomerulus

Under conditions of sustained hyperglycaemia, the glomerular cells are affected by various mechanisms, which will be discussed below. These changes lead to altered structure and function in the glomerulus. In large part, similar changes take place also in the retina and the peripheral nerve, the two other locations of microvascular structures subject to pathological changes in diabetes (Kumar V et al., 1992).

In the kidney, the first observed functional change is an abnormally

increased GFR (Ditzel J et al., 1967). This change develops before any major histological change in the glomerular structure. Later in the development of diabetic nephropathy, typical histologically visible changes are seen: thickened GBM, diffuse glomerulosclerosis, nodular glomerulosclerosis (the pathognomonic Kimmelstiel-Wilson lesions), exudative lesions in the Bowman's capsule and podocyte loss (Kumar V et al., 1992; Nishi S et al., 2000). Also the intraglomerular pressure tends to rise (Hostetter TH, 1991). In a clinical setting, the most important sign of diabetic nephropathy is proteinuria which often worsens during the course of disease. Ultimately, end-stage renal failure may develop. The detection of very low but yet increased levels of albumin in urine have been used in predicting the development of overt diabetic nephropathy (Parving HH et al., 1982; Viberti GC et al., 1982; Mogensen CE et al., 1984). However, this measurement of microalbuminuria has certain limitations due to the spontaneous regression from persistent microalbuminuria back to normoalbuminuria (Hovind P et al., 2004). Moreover, up to 12% of completely normal human individuals in all ethnic groups may show sporadic albuminuria, thus more specific markers for predicting the risk of diabetic nephropathy are needed (Caramori ML et al., 2000).

2.5.2 Molecular biological and biochemical mechanisms

The histological and functional changes described above are derivatives of alterations at the molecular level. During the course of diabetic nephropathy, due to high intra- and extracellular glucose concentration, advanced glycation end-products (AGEs) are formed, various signalling pathways are activated and there is an increased flux through the polyol and hexosamine pathways (Brownlee M, 2001). See Figure 2.5 for an overview of the parallel pathologic processes involved in the development of diabetic nephropathy. Also increased oxidative stress due to intracellular hyperglycemia is suggested to influence the pathologic changes (Giugliano D et al., 1996). Consequently, alterations in the functional integrity of a multitude of proteins and also in the gene expression patterns of growth factors, cytokines and other molecules are seen. Below, the involvement of AGEs and various growth factors in the pathogenesis of diabetic nephropathy are addressed in more detail.

2.5.2.1 Advanced glycation end-products

The AGEs were long considered to be the result of a completely non-enzymatic process, in which protein glycation randomly takes place under abnormally high glucose concentrations. However, it has been shown that the intracellular dicarbonyl formation is likely a prerequisite of significant protein glycation (Degenhardt TP et al., 1998). These highly reactive dicarbonyls are formed as a result of auto-oxidation of glucose to glyoxal (Wells-Knecht KJ et al., 1995), degradation of glyceraldehyde-3-phosphate or dihydroxyacetone phosphate to methyglyoxal (Thornalley PJ, 1990), and decomposition of the Amadori product to 3-deoxyglucosone (Brownlee M, 2001). Reaction of dicarbonyls with amino groups in proteins leads to the formation of AGEs (Degenhardt TP et al., 1998). In further developed stages of AGE formation, the structure and function of affected proteins

are altered. Intracellular signalling may be modified by these changes. Equally, the function of extracellular macromolecules and proteins is distorted (Tanaka S et al., 1988; Tsilibary EC et al., 1988; Charonis AS et al., 1990). For example, Tanaka et al. showed that the molecular packing of type I collagen is expanded due to intermolecular crosslinking by AGEs (Tanaka S et al., 1988). It has now become evident that there is also at least one major receptor for AGEs mediating the adverse effects (Neeper M et al., 1992; Vlassara H et al., 1995). This receptor for AGE (RAGE) has signalling properties as well and it is found to be upregulated in human tissues susceptible to the complications of diabetes (Kim W et al., 2005), hence RAGE is suggested to contribute to the pathogenesis of diabetes and its complications. AGE formation takes place also in the circulation and altered plasma proteins are bound to endothelial AGE receptors causing endothelial dysfunction (Yan SD et al., 1994; Lander HM et al., 1997; Smedsrod B et al., 1997). RAGE has also been found to be expressed in the podocyte and in a model of diabetic nephropathy, it has been shown to be required for the mediation of the adverse effects of AGE (Wendt TM et al., 2003).

2.5.2.2 Intracellular signalling pathways and growth factors mediating the pathological changes

Intracellular hyperglycaemia leads to increased *de novo* synthesis of diacylglycerol (DAG) which is a key intracellular metabolite and among others an activator of several protein kinase C (PKC) isoforms, most importantly the PKC-β isoforms (Koya D et al., 1998). Other pathways of PKC activation are also suggested, such as AGE receptor –ligation and increased activity of the polyol pathway due to activation of aldose reductase (Ramana KV et al., 2005). This leads to a great number of pathologic changes, such as increased production of endothelial nitric oxide synthase (eNOS), endothelin-1, vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), plasminogen activator inhibitor 1 (PAI-1), nuclear factor kappa B (NF-κB), and the activation of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) -oxidases (Brownlee M, 2001). As the renin-angiotensin system (RAS) gets activated in a patient with type 1 diabetes, angiotensin II levels increase (Ganong WF, 2003a). The activation of the angiotensin receptor in the mesangial cells is known to further stimulate TGF-β production and extracellular matrix (ECM) production (Wolf G et al., 1997). Since TGF-β is a secreted growth factor, it effects also on the podocyte in a paracrine manner.

The TGF-β seems to play a central role as a mediator in the pathologic changes in the glomerulus. It has been shown that the AGE formation, PKC activation, angiotensin II, and shear stress increase TGF-β expression (Gnudi L et al., 2003). TGF-β is a potent growth factor promoting the deposition of ECM components, such as collagen I, IV and fibronectin. This leads to the histologically evident glomerular expansion and thickening of the basement membrane. The effects of TGF-β are mediated by the TGF-β receptor type II (Ziyadeh FN, 2004), while the Smad-pathway is the downstream intracellular signalling pathway involved in TGF-β signalling (Wang W et al., 2005). Also the connective tissue growth factor (CTGF)

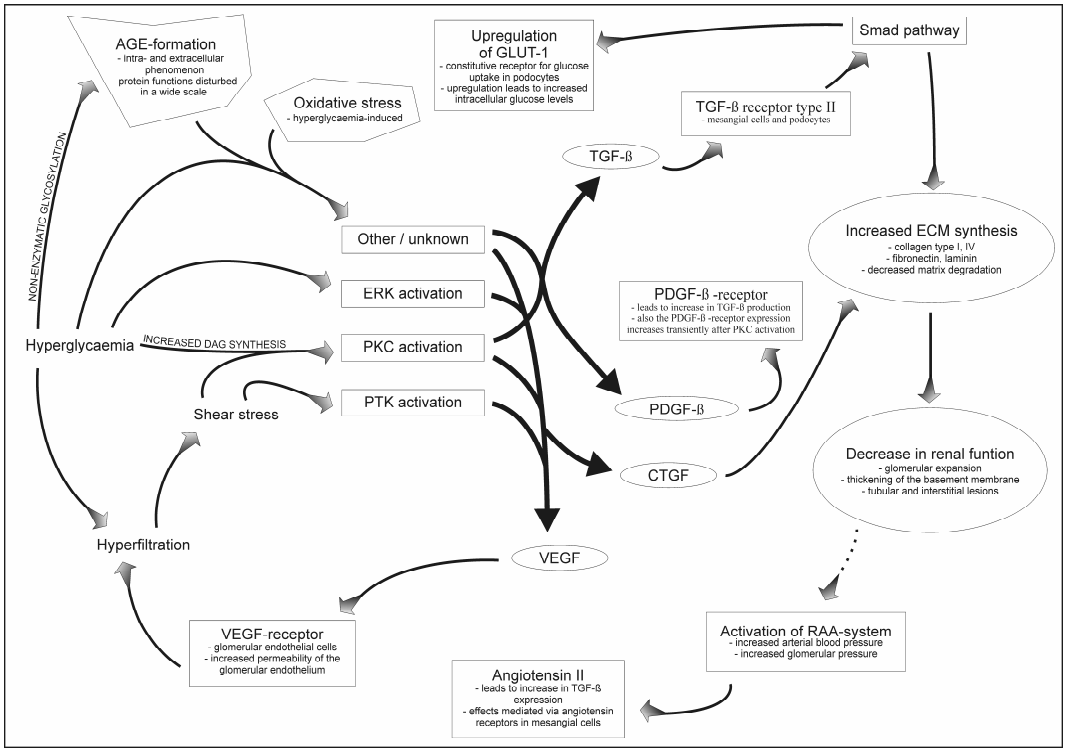


Figure 2.5: An overview of the signalling pathways involved in the pathogenesis of diabetic nephropathy.

stimulates ECM synthesis. The increase in ECM synthesis induced by AGEs has recently been shown to be mainly mediated by the CTGF (Zhou G et al., 2004) in rat mesangial cells. TGF-β also upregulates the expression of GLUT-1, the cell surface receptor for glucose in renal cells (Inoki K et al., 1999). Thus, glucose influx increases and the intracellular glucose-induced metabolic abnormalities are accelerated.

Another important mediator seems to be the VEGF. Hyperglycaemia increases VEGF excretion in the mesangial cell and podocyte via pathways involving PKC and extracellular signal-regulated kinase (ERK) (Kim YH et al., 2001; Hoshi S et al., 2002). Receptors for VEGF in the glomerulus are found in the endothelial cells and it is thought that this growth factor increases the permeability of the glomerular endothelium and is therefore responsible for the hyperfiltration seen in early diabetic nephropathy. Also mechanical stretch mimicking the shear stress caused by hyperfiltration and increased glomerular pressure increased the excretion of VEGF in the mesangial cells. In a study demonstrating this effect it seemed that the effects of shear stress in mesangial cells are mediated via a pathway dependent on PKC and protein tyrosine kinase (PTK) since the combined inhibition of these enzymes completely prevented the increased VEGF excretion in an *in vitro* experiment (Gruden G et al., 1997).

A fourth growth factor, the platelet derived growth factor beta (PDGF-β) is also involved in the histological alterations in the

glomerulus. Under high glucose concentrations the PDGF-β growth factor and the corresponding receptor are upregulated in the mesangial cell leading to later increase in TGF-β expression (Di Paolo S et al., 1996).

2.5.3 Genetic risk factors for diabetic nephropathy

Support for the existence of hereditary susceptibility to diabetic nephropathy emerges from epidemiologic observations. Studies of families of patients with diabetic nephropathy have shown an increased risk in the relatives (Seaquist ER et al., 1989). Also, the differences between various ethnic groups in development of diabetic nephropathy support the genetic aspect. Examples of this are the Pima Indians, who in addition to having a high incidence of type 2 diabetes, have an elevated risk for diabetic nephropathy as well (Pettitt DJ et al., 1990). In families of African American patients with type 2 diabetes -induced end-stage renal disease the relatives have an eightfold risk of developing diabetic nephropathy, as compared to the relatives of a type 2 diabetic control population without renal complications (Freedman BI et al., 1995). Also, in a Finnish material the presence of type 2 diabetes in the parents of a type 1 diabetic patient affected the risk of diabetic nephropathy (Fagerudd JA et al., 1999). This effect could be due to genetic and/or environmental factors passed on in the family.

Efforts to identify the genetic factors modulating the risk for diabetic nephropathy have not yielded much result. However, some candidate genes have been identified and several other susceptibility loci are proposed to associate with diabetic nephropathy. Candidate genes studied include the ACE gene, endothelial nitric oxide synthase gene (*NOS3*), angiotensinogen, angiotensin II type 1 receptor, apolipoprotein E and TGF-β (Merta M et al., 2003). Also single-nucleotide polymorphisms in the promoter region of the PKC-β1 gene are suggested to associate with the development of diabetic nephropathy (Araki S et al., 2003). Other suggested functional candidates include the aldose reductase gene *AKR1B1* and carnosinase genes *CNDP1* and *CNDP2* (Sale MM et al., 2006). Carnosinase has a function as a scavenger of reactive oxygen species, and thus reduces the formation of AGEs. As diabetic nephropathy is considered to be a complex disease with a polygenic background, candidate gene studies are often unsuccessful or too unreliable, whilst genetic linkage studies have been difficult to perform due to the lack of extended pedigrees (Lindner TH et al., 2003). With special reference to the podocyte proteins, a linkage analysis of 212 sibling pairs excluded the association of nephrin, podocin, CD2AP or the Wilms tumor loci with diabetic nephropathy (Iyengar SK et al., 2003).

2.5.4 Immunological studies on mechanisms of diabetic nephropathy

2.5.4.1 Inflammation

Recently increased levels of inflammatory markers such as C-reactive protein and interleukin-6 were found to be present in type 1 diabetic patients with micro- or macroalbuminuria (Saraheimo M et al., 2003). The origin of the inflammation remains unclear. However, this finding

suggests that the pathogenetic mechanisms of diabetic nephropathy also involve the immunological and inflammatory process. This mechanism is further supported by the finding that diabetic mice deficient in intercellular adhesion molecule 1 (ICAM-1), a molecule mediating macrophages to diabetic kidney, are protected against renal injury (Okada S et al., 2003). It is also known that the activation of PKC in the mesangial cell induces the expression of ICAM-1 (Park CW et al., 2000). This links the metabolic disturbances and alterations in intracellular signalling in diabetes to inflammation (Tuttle KR, 2005).

2.5.4.2 Oxidized LDL autoantibodies

Increased oxidative stress due to hyperglycaemia is suggested to be involved in the pathogenesis of diabetic nephropathy, as discussed earlier in this chapter. It is known that oxidized low-density lipoprotein (LDL) is immunogenic and forms immunocomplexes with corresponding antibodies and that these immunocomplexes are atherogenic and promote inflammation (Lopes-Virella MF et al., 1996). Therefore, the possible role of oxidized LDL in development of diabetic complications has been studied and several independent authors have reported that there seems to be no relation between oxidized LDL autoantibodies and albuminuria (Korpinen E et al., 1997; Leinonen JS et al., 1998; Willems D et al., 1998). However, in 2002 it was found out that a high concentration of these immunocomplexes formed of antibodies with high avidity does indeed relate to proteinuria in diabetic patients (Atchley DH et al., 2002).

2.5.4.3 Other factors

Studies on other autoantibodies present in patients with diabetes, such as phospholipid and GADA have shown no correlation between these autoantibodies and nephropathy in diabetes (Roll U et al., 1995; Vinik AI et al., 1995). Sulphatide is a preferentially neural epitope found also in the islets of Langerhans and in the kidneys of diabetic BB rats (Buschard K et al., 1993). Anti-sulphatide antibodies have been suspected to be involved in the pathogenesis of diabetic complications but the available data do not give further support to this hypothesis. Recently, the immunological pathogenetic aspect has been supported by the finding that circulating immune complexes of IgG antibodies are associated with early diabetic nephropathy (Nicoloff G et al., 2004). Already in 1972, linear deposits of immunoglobulin along with complement in glomeruli of diabetic rats were detected (Mauer SM et al., 1972). This has been verified by another study published in 1981 (Axe SR et al., 1981). The exact pathogenic function of these immunoglobulin deposits in diabetic glomeruli is not clear, although it is evidence of the involvement of immunological factors at some stage in this complication.

2.6 Proteins of the podocyte foot process

A multitude of podocyte proteins relevant to the filtration slit function has recently been discovered. The protein components of the foot process are listed in Table 2.6., and a schematic representation of these proteins in the podocyte foot process is shown in Figure 2.6.1.

2.6.1 Apical foot process membrane

The apical side of the podocyte foot process is facing the urinary space. On the apical membrane, podocalyxin is an important transmembrane protein with a strongly glycosylated and negatively charged extracellular part (Kerjaschki D et al., 1984). Podocalyxin molecules are thought to contribute to keeping the filtration pores open by holding up anionic repulsion between the opposing foot processes. Podocalyxin is connected to the actin cytoskeleton via the Na^+/H^+ exchanger regulatory factor 2 (NHERF-2) and ezrin (Takeda T, 2003). The importance of podocalyxin to the structure of the foot process is corroborated by the phenotype of podocalyxin knock-out mice, which present with anuria and perinatal lethality. In the glomeruli of these mice podocytes are present but lack foot processes (Doyonnas R et al., 2001).

Glomerular epithelial protein-1 (GLEPP1) is a membrane protein-tyrosine phosphatase (PTPase) with a large extracellular domain containing eight fibronectin type III-like repeats, a hydrophobic transmembrane segment, and a single PTPase domain (Thomas PE et al., 1994). It is suggested that GLEPP1 regulates the glomerular filtration via effects on the podocyte structure and function, since mice deficient in GLEPP1 have a decreased GFR, a low number of podocyte foot processes and reduced expression of nephrin (Wharram BL et al., 2000).

Megalin is a multiligand endocytic receptor protein of the low-density lipoprotein receptor-related protein (LRP) family found all over the podocyte cell membrane in murine glomeruli. Megalin seems to be involved in the uptake of lipoproteins from the extracellular space (Kerjaschki D et al., 1983; Kerjaschki D et al., 1997). It also has a wide variety of other ligands, such as 25-OH vitamin D (Leheste JR et al., 2003), and in the kidney megalin is mainly expressed at the epithelium of the proximal tubules where it is a highly active endocytic protein. Megalin is also present at the basal aspect of the podocyte foot process. It should be noted, however, that so far megalin has not been shown to be expressed in the human podocyte. The membrane-associated guanylate kinase inverted protein 1 (MAGI-1) has been shown to bind megalin (Patrie KM et al., 2001). MAGI-1 seems to be linked to the cytoskeleton and it is expressed in the human kidney where it closely parallels the expression of GLEPP1. MAGI-1 interacts with various proteins and it is proposed to function as a scaffolding protein.

The junctional adhesion molecule 4 (JAM4) is also localized to the apical

membrane close to ezrin. JAM4 interacts with MAGI-1 and it has been suggested to have a different role at the apical podocyte foot process besides the role as a junctional molecule (Harita Y et al., 2005).

2.6.2 Lateral podocyte membrane

2.6.2.1 Nephrin and other cell adhesion-associated molecules

Nephrin gene *NPHS1* is located in the human chromosome 19q13.1. Nephrin is a transmembrane protein with 1241 amino acids, and it belongs to the immunoglobulin superfamily of cell adhesion molecules. Nephrin contains one fibronectin-like domain, eight Ig-like domains and a short intracellular part. Due to the presence of several tyrosine residues in the intracellular domain, nephrin has signalling capabilities. The congenital nephrotic syndrome of the Finnish type (CNF) is caused by mutations in *NPHS1* (Kestilä M et al., 1998). Nephrin was the first identified structural protein of the podocyte slit diaphragm (Ruotsalainen V et al., 1999). Nephrin mRNA is known to be alternatively spliced to one additional form in the human (Holthöfer H et al., 1999a) and to four additional splice variants in the rat (Ahola H et al., 1999). One of these, the variant nephrin-α, is lacking the exon 24 encoding the transmembrane part, and could therefore translate into a soluble form of nephrin. The role of these splicing variants has not been conclusively established. Along with the expression in the kidney, nephrin expression has been also revealed in the pancreas (Palmen T et al., 2001; Zanone MM et al., 2005), brain (Putaala H et al., 2001), testis (Liu L et al., 2001) and placenta (Beall MH et al., 2005). However, a contradictory report states that nephrin expression is restricted to kidney, and that CNF patients devoid of nephrin do not seem to suffer from major clinical symptoms referring to the above mentioned organs (Kuusniemi AM et al., 2004). As already described in chapter 2.5., the PKC activity is increased in the glomerular mesangial cells as well as in the podocytes. It has been shown that the expression of nephrin mRNA in the podocyte increases with the activation of PKC (Wang SX et al., 2001b). Interestingly, human podocytes seem to be responsive to regulation by insulin (Coward RJ et al., 2005b) and nephrin appears to have a critical role for this action (Coward RJ et al., 2005a).

In addition to nephrin, a set of other adhesion proteins have been localized to the podocyte filtration slit. Neph1, -2 and -3 are three closely related transmembrane proteins of the immunoglobulin superfamily, with 5 extracellular Ig-type domains and an intracellular part with signalling capabilities (Ihalmo P et al., 2003; Sellin L et al., 2003). To avoid misunderstandings, it should be noted that Neph3 is also known as filtrin or KIRREL-2. The Neph-proteins have substantial homology to nephrin and they are all shown to interact with podocin. The extracellular parts of Neph1 (Barletta GM et al., 2003) and Neph2 (Gerke P et al., 2005) interact with nephrin as well. Similarly to nephrin, Neph1 is crucially important to the structure and function of the podocyte

Table 2.6: Principal protein components at the membranes of podocyte foot process

Membrane area	Protein	Description	Reference
Apical	Podocalyxin	Transmembrane protein keeping the filtration pores open	Kerjaschki D et al., 1984
	NHERF-2	Intracellular protein linking podocalyxin to the cytoskeleton	Takeda T, 2003
	Ezrin	Intracellular protein linking podocalyxin to the cytoskeleton	Takeda T, 2003
	GLEPP1	Transmembrane protein possibly regulating glomerular filtration	Thomas PE et al., 1994
	Megalin	Multi-ligand transmembrane receptor molecule	Kerjaschki D et al., 1983
	MAGI-1	Intracellular cytoskeleton-linked protein interacting with megalin and JAM4	Patrie KM et al., 2001
	JAM4	Transmembrane junctional molecule with unknown function at the apical membrane	Harita Y et al., 2005
	TRPC6	Cation-channel at cell surface important for renal function	Reiser J et al., 2005
Lateral	Nephrin	Transmembrane cell adhesion molecule important for the slit diaphragm	Ruotsalainen V et al., 1999
	Neph1/2/3	Three closely related transmembrane proteins contributing to the slit diaphragm	Ihalmo P et al., 2003; Sellin L et al., 2003
	Densin	Transmembrane adhesion molecule suggested to maintain podocyte cell membrane polarity	Ahola H et al., 2003
	P-cadherin	Transmembrane adhesion molecule located to the slit diaphragm	Reiser J et al., 2000
	Catenins	Intracellular binding partners of P-cadherin, linking it intracellularly to ZO-1 and FAT	Reiser J et al., 2000
	FAT	Transmembrane molecule contributing to the slit diaphragm	Inoue T et al., 2001
	MEGF1	Transmembrane molecule contributing to the slit diaphragm	Sun Y et al., 2005
	VASP	Intracellular protein binding to FAT1 and actin cytoskeleton	Moeller MJ et al., 2004
	Podocin	Integral transmembrane protein interacting with nephrin, Neph-proteins and CD2AP	Roselli S et al., 2002
	CD2AP	Cytoplasmic protein interacting with nephrin and podocin to facilitate signalling	Shih NY et al., 2001
	ZO-1	Large cytoplasmic protein binding Neph-proteins	Kawachi H et al., 1997
Basal	Dystroglycans	Anchors the podocyte to the basement membrane	Raats CJ et al., 2000
	Utrophin	Connects the dystroglycan complex to the cytoskeleton	Raats CJ et al., 2000
	α3β1-integrin	Anchors the podocyte to the basement membrane	Regoli M et al., 1997
	Podoplanin	Transmembrane protein maintaining foot process in shape	Matsui K et al., 1998

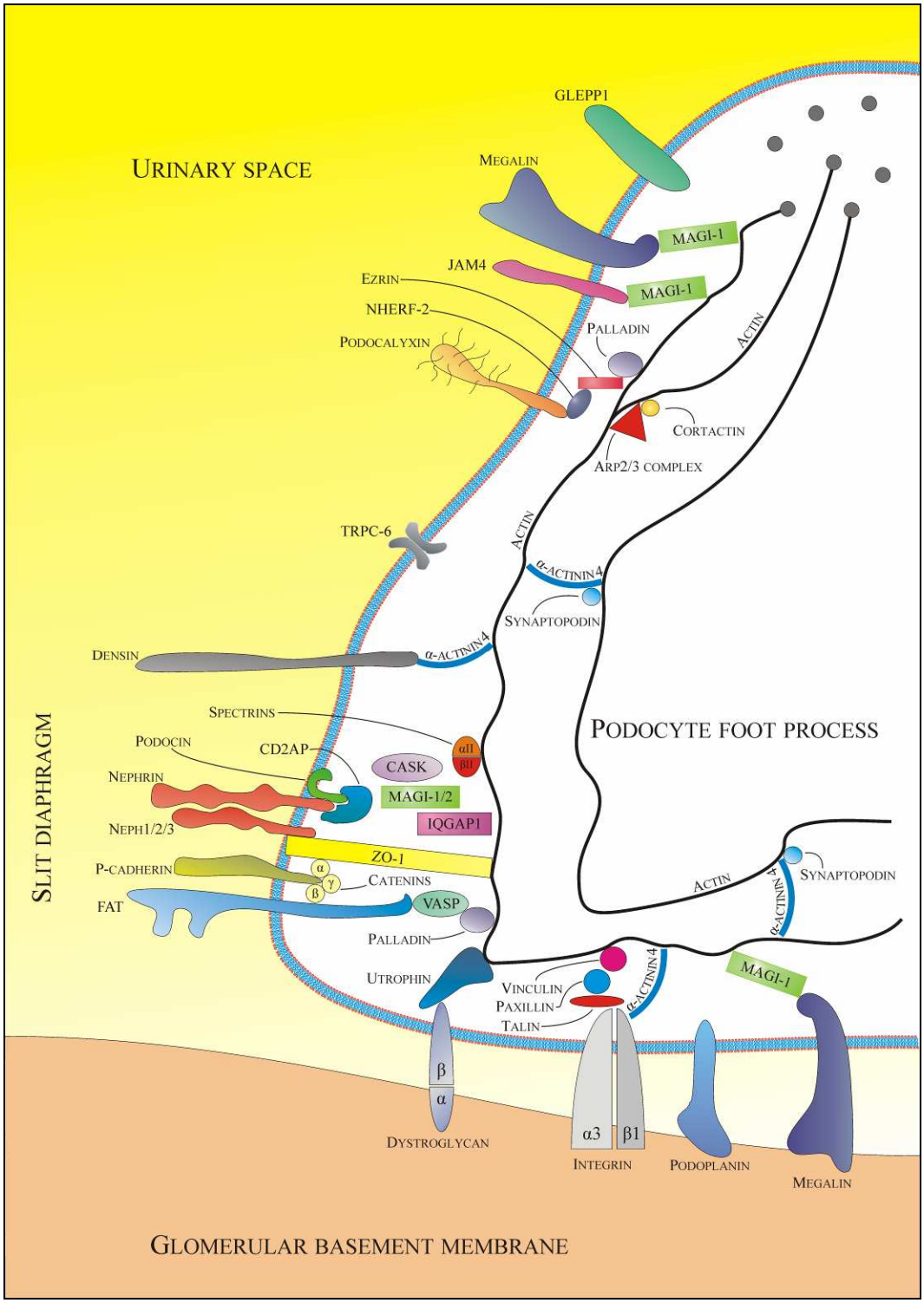


Figure 2.6.1. A schematic representation of the proteins of the podocyte foot process.

filtration slit as mice deficient in Neph1 suffer from proteinuria and perinatal lethality (Donoviel DB et al., 2001).

Densin is a transmembrane protein belonging to the leucine-rich repeats and PDZ -family of proteins (LAP) with a domain configuration similar to several adhesion molecules (Apperson ML et al., 1996). It is localized in the brain in neuronal dendrites at synapses where it is known to have interactions with alpha-actinin-4 (Walikonis RS et al., 2001). LAP proteins are suggested to maintain cell polarity (Bilder D et al., 2000). In the podocytes densin has been shown to be expressed at the slit diaphragm, and based on the role of densin in the neuronal cells it is proposed that it maintains podocyte apical-basal polarity (Ahola H et al., 2003).

The adhesion molecule P-cadherin and proteins alpha-, beta- and gamma-catenin have been found in cultured podocytes (Reiser J et al., 2000) and at the slit diaphragm (Xu ZG et al., 2005). They are all associated with the adherens-type cell junction. However, there is also one incongruous report of P-cadherin and alpha- and beta-catenin expression in the rat, where immunostaining in the podocyte was seen only in the developing kidney (Yaoita E et al., 2002). The expression of P-cadherin is known to decrease in STZ rats already at 6 weeks, suggesting its involvement in the pathological changes of early diabetic nephropathy (Xu ZG et al., 2005).

FAT is a novel member of the cadherin family, and it is shown to be a component of the slit diaphragm (Inoue T et al., 2001; Yaoita E et al., 2002). FAT is a large molecule of approximately 4600 amino acid residues that has a long extracellular domain. According to recent publications it is evident that another large protein of the same family, known as the protein 1 with multiple EGF-like domains (MEGF1/Fat2), is present at the slit diaphragm as well (Sun Y et al., 2005). The intracellular part of FAT is shown to bind to vasodilator-stimulated phosphoprotein (VASP) and is necessary for cell polarization and actin cytoskeleton assembly (Moeller MJ et al., 2004). VASP takes part in the actin cytoskeleton organization together with palladin, an actin-binding cytoplasmic protein (Boukhelifa M et al., 2004). Palladin is a protein associated to actin filament assembly and it may interact with ezrin (Mykkanen OM et al., 2001).

2.6.2.2 Scaffold proteins and signal transducers at the slit diaphragm

The intracellularly important binding partners of nephrin seem to be podocin and CD2AP. Podocin is located at the podocyte filtration slit area (Roselli S et al., 2002) and it belongs to the stomatin family of proteins. Stomatins are integral transmembrane proteins with intracellular amino- and carboxytermini (Schwarz K et al., 2001). The gene encoding podocin, *NPHS2*, is mutated in familial and sporadic focal segmental glomerulosclerosis (Boute N et al., 2000; Karle SM et al., 2002) and in some patients with a phenotypic CNF without mutations in nephrin (Koziell A et al., 2002). As mentioned above, podocin interacts with the Neph-proteins, nephrin and CD2AP, and it is suggested that it provides a scaffold for the nephrin-associated protein complex in the podocyte slit diaphragm. Similar to other stomatins, podocin is associated with lipid rafts (Schwarz K et al., 2001) and recruits nephrin and Neph-proteins to these lipid rafts to facilitate nephrin signalling (Huber TB et al., 2001; Huber TB et al., 2005). The

member 6 of the transient receptor potential cation channel subfamily C (TRPC6) is a cation-channel localized to podocyte cell membranes (Reiser J et al., 2005). Mutation in the channel is another causative factor for familial focal segmental glomerulosclerosis (Winn MP et al., 2005). The possible linking mechanism for TRPC6 and podocin to cause focal segmental glomerulosclerosis is not known. However, recent work suggests that the regulation of the TRPC6 might be associated with normal nephrin function in the podocyte (Trouet D et al., 2005). CD2AP is an 80 kilodalton (kDa) cytoplasmic protein connected to the intracellular C-terminal domain of nephrin via its C-terminal domain (Shih NY et al., 1999; Shih NY et al., 2001). CD2AP has been widely studied in association to T-cell contacts, where it is important for the cytoskeletal polarity and receptor patterning (Dustin ML et al., 1998). It is important to the signalling function of nephrin as well.

Nephrin-associated signalling is depicted in Figure 2.6.2. Nephrin is associated with lipid rafts which are signalling-associated microdomains on the cell membrane. When these lipid rafts were disrupted by antibodies against a podocyte-specific lipid raft component, the foot processes effaced and nephrin was phosphorylated and dislocated to the apical area of the foot process membrane (Simons M et al., 2001). Similarly nephrin antibodies disrupt the cell-cell connections (Khoshnoodi J et al., 2003) and cluster nephrin, which leads to phosphorylation of nephrin by Src kinases (Lahdenpera J et al., 2003). In particular, the Src kinase family member Fyn has been shown to phosphorylate the cytoplasmic end of nephrin and intensify the binding of nephrin with podocin (Li H et al., 2004). Furthermore, the action of Fyn kinase on nephrin enables the binding of phosphoinositide 3-OH kinase (PI3K) to the intracellular nephrin-podocin-CD2AP complex (Huber TB et al., 2003a). PI3K is a heterodimer comprising a regulatory subunit p85 and a catalytic subunit p110. The p85 recognizes the phosphorylated nephrin and allows the p110 to phosphorylate phospholipids at the inner leaflet of the lipid bilayer stimulating the serine-threonine kinase AKT. Further downstream phosphorylation occurs, and one phosphorylated protein is Bad, a proapoptotic factor. Phosphorylation of Bad protects the podocyte from apoptosis induced by detachment (Huber TB et al., 2003a), see also Figure 2.6.2.

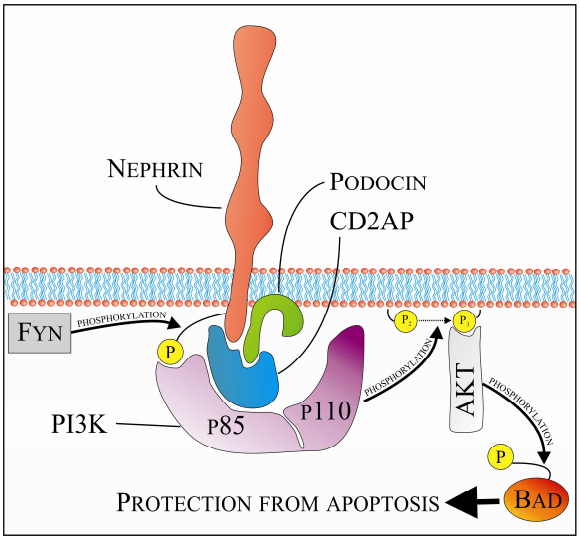


Figure 2.6.2. Nephrin-associated signalling as suggested by Li (Li H et al., 2004) and Huber (Huber TB et al., 2003a). See text for details.

Other intracellular interaction partners for nephrin include MAGI-1, which is also found to bind with the carboxyterminal end of nephrin and is localized to the slit diaphragm (Hirabayashi S et al., 2005). Also, the scaffolding proteins MAGI-2, IQ motif-containing GTPase-activating protein 1 (IQGAP1), calcium/calmodulin-dependent serine protein kinase (CASK), alpha-actinin 4, alpha-II-spectrin and beta-II-spectrin have been found to directly or indirectly associate with nephrin (Lehtonen S et al., 2004; Lehtonen S et al., 2005). These are probably linking the cell membrane proteins to the actin cytoskeleton and facilitating further signalling. Yet another scaffolding protein of the slit diaphragm is the zonula occludens 1 (ZO-1), which is a high molecular mass phosphoprotein of 225 kDa located on the cytoplasmic aspect of the slit area (Schnabel E et al., 1990). ZO-1 is a known protein component of the tight junctions, although in the podocyte it apparently does not lead to tight junction assembly. The carboxyl terminus of the Neph-proteins bind to ZO-1 (Huber TB et al., 2003b).

2.6.3 Basal podocyte membrane

The basal aspect of the podocyte is attached to the underlying basement membrane by multiple proteins forming contacts to the components of the GBM, such as laminin, agrin, biglycan, syndecan-4 and glypican-1 (Pyke C et al., 1997). Alpha- and beta-dystroglycans are present in normal renal tissue (Regele HM et al., 2000) and link the podocyte cytoskeleton to the GBM extracellularly via agrin and laminin and intracellularly via utrophin (Raats CJ et al., 2000). In contrast to dystroglycan complexes found in the skeletal and smooth muscle cells the epithelial dystroglycan complex in the podocytes does not associate with any of the known sarcoglycans or sarcospan (Durbeej M et al., 1999). The heterodimeric adhesion molecule α3β1-integrin localizes to the basal podocyte cell membrane and its

expression is decreased early in a model of diabetic nephropathy (Regoli M et al., 1997). The α3β1-integrin anchors the podocyte to the GBM components laminin and agrin (Kerjaschki D, 2001). Intracellularly, α3β1-integrin is linked to the actin cytoskeleton via talin, vinculin and paxillin (Drenckhahn D et al., 1988) and in addition directly by binding of alpha-actinin to the cytoplasmic domain of β1-integrin (Otey CA et al., 1993). Podoplanin is a 166-amino-acid transmembrane protein that apparently has a function in upholding the foot process structure and function (Breiteneder-Geleff S et al., 1997; Matsui K et al., 1998). Megalin, as already described above, is present at the basal podocyte membrane as well.

3 AIMS OF THE STUDY

This thesis aimed at the following:

(i) To study the changes in nephrin mRNA expression in experimental nephrosis.

(ii) To study the expression of nephrin in the early stages of diabetic nephropathy.

(iii) To specify the nephrin expression levels in later diabetic nephropathy.

(iv) To test whether anti-proteinuric therapies could affect the nephrin expression and other pathological changes in experimental diabetic nephropathy.

(v) To find out if nephrin could act as an autoantigen in patients with type 1 diabetes, and

(vi) whether such autoantibodies have an effect on the pathogenesis of diabetic nephropathy.

4 MATERIALS AND METHODS

4.1 Human study subjects and experimental animal models

4.1.1 Animal models

The animal models used in this work were the PAN rat (I), NOD mice (II) and STZ rat (II and III). The experimental design of each study is summarized in Table 4.1. The details of model induction, monitoring, experimental preparations and sample collection are described in the respective articles. It should be noted here that due to previous erroneous information, the time points for the STZ rat sacrifices in study II were indeed “4”, “8”, and “12” weeks, instead of the “4”, “6 and 8”, and “16” weeks indicated in the figures of the original report. Likewise, the NOD mice sacrifice time points were “3”, “6”, and “10” weeks after onset of diabetes, not after birth as stated in study II. Corrected time point designations are used throughout this thesis and an erratum has been sent to *Laboratory Investigation*.

Table 4.1: Experimental animal models

Study	Model type	Groups by time at sacrifice	n	Samples collected for analysis
I	PAN rat	PAN 3 days	6	*(i)* kidney tissue
		PAN 10 days	6	*(ii)* urine
		control rats 10 days	6	
II	NOD mice	NOD 3 weeks diabetic	3	*(i)* kidney tissue
		NOD 6 weeks diabetic	3	
		NOD 10 weeks diabetic	3	
		control mice[a]	3	
	STZ rat	STZ 4 weeks	2	*(i)* kidney tissue
		STZ 8 weeks	4	*(ii)* urine
		STZ 12 weeks	5	*(iii)* blood
		control rats 14 weeks	3	
III	STZ rat	STZ 1 week	7	*(i)* kidney tissue
		control 1 week	7	*(ii)* urine
		STZ 24 weeks	8	*(iii)* blood
		STZ + perindopril 24 weeks	8	
		STZ + aminoguanidine 24 weeks	8	
		control 24 weeks	8	

[a]The age of control mice at the time of sacrifice is not available.

4.1.2 Patients with type 1 diabetes

The patient cohort of study IV comprised 66 children and adolescents (43 male, 23 female) diagnosed with type 1 diabetes at the Department of Pediatrics, University of Oulu, Finland in a consecutive series between 1983 and 1986. The mean age at diagnosis was 8.2 years (range 0.9 to 15.6 years). Serum samples from the patients were taken at diagnosis and 2, 5 and 10 years later. All samples were stored at –20 °C until analyzed. Detailed clinical data were available including clinical history, total glycated hemoglobin, HLA-DR antigen serotypes and levels of four diabetes-associated autoantibodies, islet cell antibodies (ICA), insulin autoantibodies (IAA) or antibodies (IA; antibodies to exogenous insulin), GADA and IA-2A, in the same samples from which nephrin autoantibodies were quantified. Microalbuminuria was defined as albumin excretion ranging from 30 to 300 mg/24h and proteinuria as a rate exceeding 300 mg/24h. At the time of this study, 10 patients had been lost to follow-up. Of these, nine patients had moved to another region and one had died with a diagnosis of multiple sclerosis. The mean duration of diabetes when lost to follow-up was 8.7 years (range 4 to 13 years). The study was approved by the ethics committee of the University of Oulu. The participants and/or their parents gave appropriate written informed consent.

4.1.3 Control subjects

The control group of study IV comprised 96 non-diabetic, unselected healthy subjects (48 male, 48 female) with a mean age of 11.3 years (range 0.5 to 18.2 years). These control samples, gathered between 1989 and 1994 from children participating in a control study program or a separate school child study, were used to set the cut-off limit for our assay. An appropriate written informed consent was obtained.

4.2 Molecular biological methods

4.2.1 RNA isolation and complementary DNA synthesis

In studies I and II, total cortical ribonucleic acid (RNA) was extracted from the frozen pieces of kidney tissue (50–150 mg) with a single-step acid guanidium thiocyanate-phenol-chloroform procedure using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA was then incubated with RNase free DNase I (Promega) for 30 minutes in 37° C together with human placental RNase inhibitor (Promega) to remove traces of genomic DNA. Complementary DNA (cDNA) was synthesized with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT; Promega) from 10 µg of DNase-treated total RNA in the presence of oligo dT_{15}-primer

(Boehringer Mannheim) and an RNase inhibitor as previously described (Holthöfer H et al., 1999b).

In study III, the frozen glomeruli extracted from the sacrificed animals and stored at -80°C were homogenized and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski P et al., 1987). The purified RNA was dissolved in sterile water, quantified spectrophotometrically and its concentration adjusted to 1 µg/µl. Four micrograms of total RNA in 10 µl of water was mixed with 1 µl (0.05 µg) random hexamers (Life Technologies) and incubated for 10 min at 70°C. After cooling on ice for 2 min, 2 µl of 10x polymerase chain reaction (PCR) buffer, 2 µl of 25 mmol/l $MgCl_2$, 1 µl of 10 mmol/l deoxyribonucleotide triphosphate (dNTP) mix, and 2 µl of 0.1 mol/l dithiothreitol (DTT) were added sequentially. Tubes were incubated at 25°C for 5 min, after which 1 µl of SuperScript II reverse transcriptase was added (200U; Life Technologies) and incubated at 25°C for 10 min and at 42°C for 50 min. Reactions were terminated at 70°C for 15 min before chilling on ice. One microlitre of RNase H was added to each sample at incubated at 37°C after which samples were stored at -40°C for future use. Parallel reactions for each RNA sample were run in the absence of SuperScript II (genomic controls) to assess the degree of any contaminating genomic DNA.

4.2.2 Semiquantitative RT-PCR

In study I, semiquantitative reverse transcriptase PCR (RT-PCR) was used and the results were compared with additional results obtained by real-time quantitative TaqMan PCR, as described in the next paragraph. For the detection of different nephrin splicing forms the RT-PCR method instead of a less sensitive Northern blotting technique was used (Holthöfer H et al., 1999b). The amounts of cDNA were equalized according to ß-actin levels. Sequence-specific oligonucleotide primers were designed according to the rat nephrin sequence. The primers used only anneal to the alpha splicing variant (primers RN-S3 with RN-alpha; see Table 5.1 for primer sequences) corresponding to 480 nucleotides. PCR amplifications were performed (total volume 25 µL) in the presence of 0.5µM each of the specific oligonucleotide primers, 0.2mM dNTP (Finnzymes), 10x PCR buffer with 15mM $MgCl_2$ (Perkin-Elmer) and 1U AmpliTaq polymerase (Perkin-Elmer). PCR was run 28 cycles for ß-actin primers or 32 cycles for nephrin primers (in the first cycle denaturation was 3 min at 94°C and thereafter 45 s, annealing at 56°C for 1 min and extension at 72°C for 45 s). PCR products were electrophoresed in 6% polyacrylamide gel and quantified with the National Institutes of Health Image® program as described previously (Holthöfer H et al., 1999b).

4.2.3 Real-time quantitative PCR

To quantitate nephrin expression levels in studies I, II and III, we used the real-time fluorescence based PCR detection method (TaqMan technology; PE Biosystems) (Laurendeau I et al., 1999). The TaqMan method is based on the use of a fluorescent-labeled probe designed for the sequence between the PCR primers and the exonuclease activity of Taq polymerase. The probe is labeled with a reporter dye (FAM or VIC; PE Biosystems) at its 5' end and a

quencher dye (TAMRA; PE Biosystems) at its 3' end. When the probe is hybridized to a target sequence it is intact and the reporter dye remains quenched. In each cycle of amplification, the Taq polymerase releases increasing amounts of reporter dye proportional to the number of target sequences. The fluorescence is then measured with specifically designed instrumentation. The probe used in studies I, II and III was a FAM-TAMRA-labeled probe which was designed so that it anneals between nephrin-specific primers for the amplification of rat or mouse nephrin. See Table 5.1 for primer and probe sequences. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an endogenous control in studies I and II. The GAPDH mRNA expression values for diabetic mice and rats were used to normalize the amounts of nephrin mRNA. In study III, a commercial, pre-developed control kit probe designed for the 18S ribosomal RNA and labelled with the fluorescent reporter dye (VIC) on the 5' end and the quencher (TAMRA) on the 3' end was used to normalize the PCR results to a housekeeping gene (PE Biosystems). The real-time quantitative RT-PCR was performed using appropriate instrumentation according to the manufacturer's instructions (PE Biosystems). The derived normalized values are the averages of at least three runs.

Table 5.1: Oligonucleotide primers and probes used

Primer/probe name	Description	Sequence	Used in study
RN-S3 (forward)	Rat nephrin primer	5’-agcctcttgaccatcgctaa-3’	I
RN-alpha (reverse)	Rat nephrin primer	5’-gtcctcgccttcagcacctg-3’	I
2636U (forward)	Mouse nephrin primer	5’-atctccaagaccccaggtacaca-3’	II
2817L (reverse)	Mouse nephrin primer	5’-agggtcagggcggctgat-3’	II
2709U (forward)	Rat nephrin primer	5’-taatgtgtctgcggcccag-3’	I,II,III
2783L (reverse)	Rat nephrin primer	5’-ttggtgtggtcagagccaag-3’	I,II,III
2735U (forward)	Murine nephrin probe	5’-FAM-ccctcttcaaatgcacggccacca-TAMRA-3’	I,II,III
2316 (forward)	Human nephrin primer	5’-tcgaagcttgccgccaccatggtcaactgggagagactg-3’	IV
3723 (reverse)	Human nephrin primer	5’-tacgtagaattcttacaccagatgtcccctcagctcgaa-3’	IV

4.2.4 *In situ* hybridization

For the direct detection of nephrin mRNA in study III, an anti-sense riboprobe was generated as previously described (Gilbert RE et al., 2000). In brief, a 293 base pair cDNA coding for rat nephrin was cloned into pGEM-T (Promega), linearized with Not I and an anti-sense riboprobe was produced using T7 RNA polymerase. The purified riboprobe length was adjusted to approximately 150 bases by alkaline hydrolysis. Four-µm-thick sections of rat kidney obtained from the experimental procedures in study III were cut fixed on slides precoated with 3-aminopropyltriethoxysilane and baked overnight at 37°C. Tissue sections were dewaxed and rehydrated, and in situ hybridization was performed using radiolabelled riboprobe as previously described (Gilbert RE et al., 2000).

Following hybridization, slides were washed, dehydrated in graded ethanol, air dried, and exposed to Kodak X-Omat autoradiographic film for 3 days. Slides were then dipped in Ilford K5 nuclear emulsion, stored in a light-free box with desiccant at 4°C for 21 days, immersed in Kodak D19 developer, fixed in Ilford Hypan, and stained with haematoxylin and eosin.

4.3 Immunological analysis

4.3.1 Immunoblotting

For the immunoblots in studies I and II, urine samples were loaded onto 8% sodium dodecyl sulphate polyacrylamide gels with reducing loading buffer. Proteins were electrophoresed with reducing conditions and electrotransferred to a nitrocellulose membrane as described previously (Reivinen J et al., 1994). To prevent nonspecific binding, the membrane was blocked by incubation in 3% bovine serum albumin. Primary polyclonal rabbit anti-nephrin antibody directed at the extracellular part of nephrin (Ahola H et al., 1999), used at 1:300 (diluted in phosphate buffered saline with 0.1% of the soluent Tween-20; PBS/0.1% Tween-20), was applied for 60 minutes at room temperature. After several rinses with PBS/0.1% Tween-20, a secondary antibody (Horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins; Dako) was used at 1:2000 and imaging with an enzymatic chemiluminescence (ECL) substrate was done with appropriate instrumentation.

4.3.2 Immunohistochemistry

For the immunohistochemical stains in study II, sagittally cut kidneys were fixed in 4% paraformaldehyde (in PBS) and embedded in paraffin. For stainings, 3 µm kidney sections were cut and deparaffinized, rehydrated, microwave-antigen unmasked, and incubated with 3% hydrogen peroxide methanol to block endogenous peroxidase activity. After several washes in PBS, the sections were blocked with 10% nonimmune goat serum. The primary polyclonal antibodies used were against the extracellular part of nephrin (dilution 1:50 and incubation 60 minutes). After a thorough rinsing with PBS/0.05% Tween-20 the sections were incubated for 10 minutes in biotinylated secondary antibody against rabbit immunoglobulins. The sections were then incubated in streptavidin-peroxidase after mixing with substrate-chromogen. Slides were counterstained with hematoxylin and mounted. All stages were performed according to instructions for peroxidase staining with a Histostain-DS kit (Zymed Laboratories).

In study IV, normal rat kidney tissue was obtained from our previous experiments (Luimula P et al., 2002). Animals were treated according to the rules and regulations of the Ethical Committee of the University of Helsinki. Rat kidney tissue instead of human kidney tissue was chosen to avoid unspecific staining possibly given by human

serum samples. The 6 µm thick rat cortex cryostat sections were air-dried, fixed in 4% parafolmaldehyde in PBS and then blocked with CAS-Block (Zymed Laboratories). The sections were incubated with positive control antibody, patient serum or control serum diluted 1:20, 1:50 or 1:100 in ChemMate Antiboby Diluent (Dako) over night at 6°C. We used a polyclonal protein-A-purified rabbit antibody for intracellular rat nephrin as the positive control (Luimula P et al., 2000). After three washes in PBS the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (1:100; Dako) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (1:100; Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. After washing the slides were covered with Vectashield Hard Set mounting medium (Vector Laboratories). Fluorescence microscopy was performed with an Olympus BX50 microscope equipped with a cooled digital camera (Hamamatsu Photonics). Corel Photo-Paint 12.0 and CorelDraw 12.0 (Corel Corporation) software was used for image documentation.

4.4 Radioimmunoprecipitation assay for detecting nephrin autoantibodies

4.4.1 Labelled recombinant nephrin

For the preparation of the radioimmunoprecipitation assay in study IV, normal human kidney cDNA was obtained from our previous experiments, as described elsewhere (Holthöfer H et al., 1999a). PCR amplification with human nephrin specific primers (see Table 5.1 for detailed sequences) was used to produce the nephrin-specific insert, which was cloned under a T7 promoter sequence in the circular plasmid vector pGEM-4z (Promega). The recombinant nephrin had 470 amino acid residues spanning from the extracellular side (772 methionine; GenBank accession code AF035835) to the intracellular C-terminal end (1241 valine). ^{35}S-methionine labeled recombinant protein was produced by *in vitro* transcription – translation using the TnT T7 Coupled Reticulosyte Lysate System (Promega) and Redivue L-[^{35}S] methionine (Amersham Biosciences) according to manufacturers' instructions. Unincorporated ^{35}S-methionine was removed by gel chromatography through a NAP-5 column (Amersham Biosciences).

4.4.2 Radioimmunoprecipitation assay

The radioimmunoprecipitation assay used in study IV was performed essentially as described earlier (Savola K et al., 1998). In 96-well sample plates (Wallac), 2 µl of patient serum and 15 000 counts per minute (cpm) of labeled nephrin were incubated for 7 to 15 hours in a buffer containing 50 mM Tris, 150 mM NaCl and 0.1 percent Tween-20 (TBST; pH 7.4). Then Protein A-sepharose 4B (Zymed Laboratories) was added and incubation was continued for 1 to 2 hours. After this, the samples were washed eight times with 140 µl of TBST, scintillation cocktail (OptiPhase 'HiSafe' 3; Wallac) was added and the activity measured. Each sample was measured in

duplicate and the final values are averages of at least two measurements. A dilution series (1:25, 1:50, 1:100, 1:200, 1:400) of a polyclonal nephrin antibody (Ahola H et al., 1999) was used for standardization. These dilutions were given a relative value of 1, 0.5, 0.25, 0.125 and 0.0625, respectively. The actual test samples were given a value in relative units (RU) using the standard curve of the dilution series to diminish the effect of interassay variation on the overall levels of measured activity. The intra- and interassay coefficients of variation were 4.7% and 17.2%, respectively. In our assays, the mean level of bound nephrin label in the 96 healthy controls was 0.066 RU with a standard deviation (SD) of 0.015. The cut-off limit used was 0.10 RU and was defined as the 99th percentile of the values in the control group (mean + 2.09 SD). We selected this cut-off limit because of the background cpm in the normal control sera. Such background was also found in a similar radioligand binding assay for the detection of GADA (Grubin CE et al., 1994).

To verify the specificity of our assay, we produced non-radiolabeled recombinant nephrin and performed blocking reactions. Briefly, to the normal assay reaction containing 15000 cpm labeled nephrin, we added an amount of non-labeled recombinant nephrin equivalent to 20000, 40000 or 60000 cpm of labeled recombinant nephrin. Samples of nephrin autoantibody-positive and negative diabetic patients were tested. As a positive control we included a sample of the polyclonal nephrin antibody at 1:50 dilution. Except for the negative patient samples, the blocking reactions resulted in uniform and dose-dependent decrease of the measured mean cpm levels to approximately 30%, 14% and 0%, as compared to the respective unblocked reaction and standardized to the background cpm levels of negative patient samples or TBST controls. Addition of irrelevant luciferase control protein to the reactions did not affect the results.

4.5 Statistical analysis

In studies I and II comparing the results of the nephrin mRNA expression measurements the normalized values of nephrin mRNA are averages of three to four runs and are shown as the mean ± standard error of the mean (SEM). The significance was tested using the Student’s t test and the Mann-Whitney U test where appropriate.

In study III, because of its highly skewed distribution albumin excretion rate (AER) was logarithmically transformed before statistical analysis and expressed as the geometric mean x/÷ tolerance factor. Between group differences were analysed by the analysis of variance test (ANOVA) with correction for multiple comparisons using the Fisher's least significant difference test. Linear regression analysis was used to determine the correlation between the magnitude of nephrin gene expression and proteinuria. Analyses in study III were performed using the Statview SE+ Graphics package (Abacus Concepts).

When analysing the radiommunoassay results in study IV, the statistical analysis software (SPSS 12.0.1 for Windows; SPSS Inc.) was used. Normally distributed scale variables were compared with the use of the two-tailed independent samples t test. The nonparametric Mann-Whitney U test was applied to compare skewed variables. Categorical variables were tested with chi-square statistics. A Kaplan-Meier survival curve was constructed for the time of diagnosis of diabetes to the time of diagnosis of microalbuminuria or proteinuria. By applying multivariate tests in a general linear model we analyzed the correlation of consecutive nephrin autoantibody titers within-subjects across the follow-up time and also compared the titer profiles between groups categorized by the appearance of microalbuminuria during the follow-up period.

All given *P* values are two-sided and *P* values <0.05 were considered significant.

5 RESULTS

5.1 Changes in nephrin expression

5.1.1 Nephrosis model

In the PAN model, the expression of nephrin-alpha mRNA decreased to 40% already by day 3 after induction, and at 10 days after puromycin aminonucleoside treatment the expression was only 30% of the expression level of controls. The expression of full length nephrin mRNA decreased with close similarity (Study I, Figure 3). To verify the adequate precision of the semiquantitation method we performed an additional assay of the full length nephrin mRNA levels by TaqMan RT-PCR analysis. The values correlated well with those of the semiquantitation (Study I, Table 1).

5.1.2 Diabetic nephropathy models

5.1.2.1 Expression at the mRNA level

In study II, the nephrin mRNA levels of diabetic rats had increased approximately 50% already during 4 to 8 weeks after the STZ induction as compared with the control rats ($P<0.05$). At 12 weeks, in the SZT rats, the elevation in nephrin mRNA expression was not statistically significant as compared to the expression levels of the controls. The results of nephrin mRNA level quantifications from the STZ rats in studies II and III are compiled in Figure 5.1. A similar, statistically significant elevation of nephrin-specific mRNA level was seen in the NOD mice at 3 and 6 weeks after the onset of diabetes (Study II, Figure 6). At 10 weeks in NOD mice, the nephrin mRNA expression level was still elevated, although not significantly as compared to the control mice.

As studied in the 1 week diabetic STZ rats (study III), short-term diabetes did not alter nephrin gene expression. However, in long-term diabetic STZ rats at 24 weeks after model induction, nephrin mRNA expression was reduced to 50% compared to control animals. In perindopril-treated diabetic rats the nephrin gene expression was similar to control animals. In contrast, in the aminoguanidine-treated rats, nephrin expression was similar to untreated diabetic rats (Figure 5.1). The *in-situ* hybridization localized nephrin mRNA exclusively in the glomerulus. Nephrin mRNA was detected in a predominantly peripheral distribution consistent with expression in the podocytes in both control and diabetic rat kidneys. Fewer autoradiographic grains were detected in glomeruli from diabetic rats at 24 weeks and diabetic rats treated with aminoguanidine at 24 weeks than in the control animals, consistent with the quantitative TaqMan RT-PCR results.

5.1.2.2 Expression at the protein level

As seen by light microscopy in study II, immunohistochemical staining for nephrin in control animals revealed an epithelial-like pattern in glomeruli, with

clear reactivity in cells facing the urinary space, whereas the innermost glomerular tuft areas remained unstained (Study II, Figure 7A). After 8 and 12 weeks of STZ induction, the overall intensity of nephrin staining was higher, and altered nephrin localization was observed. Typically a more central localization and nephrin staining deposits closer to the capillaries were observed in the STZ treated rats (Study II, Figure 7B).

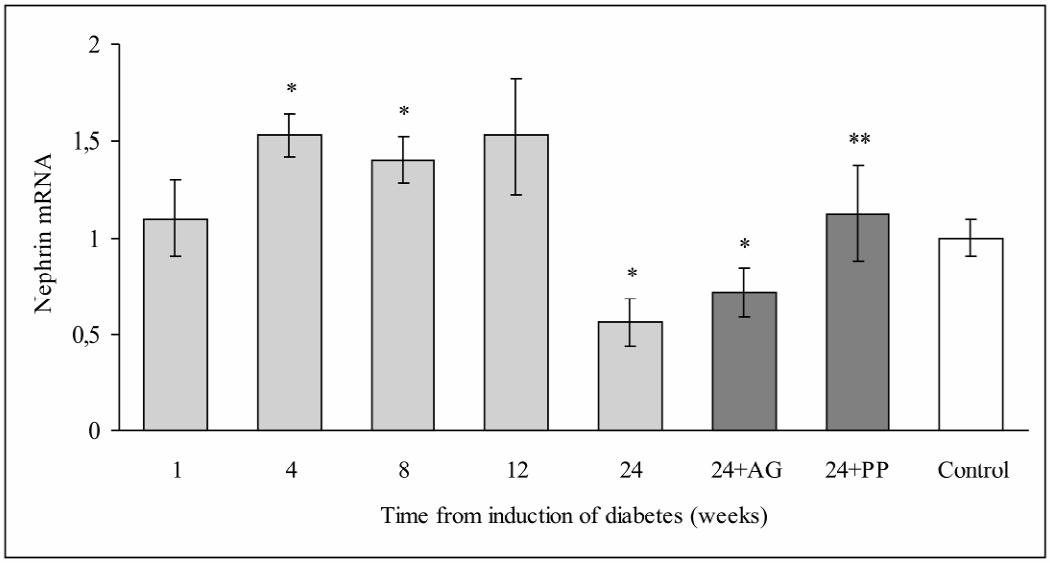


Figure 5.1. Nephrin mRNA changes in streptozotocin-treated rats. The data represented in the figure are combined from studies II and III. Values shown are means ± SEM relative to controls, which were assigned an arbitrary value of 1. The darkened bars show groups treated with either aminoguanidine (AG) or perindopril (PP). **P*<0.05 versus control. ***P*<0.01 versus the 24 weeks diabetic group. At the time of model induction, the rats were 12 (study II) or 13 (study III) weeks of age.

5.2 Urinary nephrin

In study I, the immunoblotting of proteinuric stage urine samples at 6 and 10 days from rats treated with puromycin aminonucleoside revealed a band of ~166 kDa. No nephrin-specific bands could be observed in the day 0 and day 3 urine samples of low proteinuria in the PAN model (Study I, Figure 4). Immunoblots of the urine of STZ rats in study II detected a nephrin-specific band of ≤185 kDa in samples gathered at 4 to 6 weeks after model induction (Study II, Figure 4).

5.3 Nephrin autoantibodies

5.3.1 Clinical and immunological profile of patients

Comparison of the baseline clinical data of the type 1 diabetic patients at diagnosis including age, gender, blood pressure, creatinine levels, blood glucose, glycated hemoglobin levels, blood pH, presence of ketoacidosis, C-peptide levels or initial insulin dose categorized by the presence of nephrin autoantibodies revealed no significant differences (Study IV, Table 1). A similar comparison between the respective patient characteristics categorized by the appearance of a sign of diabetic nephropathy during the follow-up period did not reveal any statistically significant differences either. During the follow-up period of up to 19 years, 11/66 (16.7%) had developed microalbuminuria and 3/66 (4.5%) had progressed to overt proteinuria. In those patients the mean duration of diabetes at the manifestation of microalbuminuria and proteinuria was 9.5 and 12.0 years, respectively. However, the time from diagnosis of diabetes to appearance of microalbuminuria/proteinuria varied considerably, with the first patient presenting with microalbuminuria already at 2 years after diagnosis (Study IV, Figure 1). In tests to determine the correlation between the antibody levels and the ICA level in respective samples we noted that nephrin autoantibody levels did not correlate with ICA levels, unlike the IA-2A and GADA levels did. Interestingly, patients presenting with nephrin autoantibodies had always a HLA-DR4 and/or HLA-DR3 antigen present as well (Study IV, Table 3).

5.3.2 Autoantibodies to nephrin

At the time of diagnosis 16/66 (24.2%) patients were positive for nephrin antibodies. These proportions were 14/61 (23.0%), 8/58 (13.8%) and 8/44 (18.2%), respectively at 2, 5 and 10 years (Study IV, Table 2). Altogether 29/66 (43.9%) patients tested positive for nephrin autoantibodies in at least one sample. Substantial fluctuation was seen in the levels of nephrin antibodies over the 10-year measurement period, and the analysis of consecutive nephrin autoantibody levels within-subjects excluded any significant correlation (P=0.14). Similarly, the comparison between subject groups defined by the appearance of diabetic nephropathy (P=0.39) showed no difference among the nephrin autoantibody level profiles. Only 4/66 (6.1%) patients were positive in all measured samples.

5.3.3 Nephrin autoantibodies and renal injury

Among the patients with signs of renal injury (microalbuminuria or proteinuria), 4/14 (28.6%) were positive for nephrin autoantibodies on at least one occasion. The respective proportion among the patients without a sign of renal complication was 25/52 (48.1%). In the subgroup of patients with renal injury and nephrin autoantibodies detected in at least one sample the mean duration of diabetes at

manifestation of microalbuminuria was 7.7 years, with a 95% confidence interval (CI) between 0.0 to 20.4 years. The patients free of nephrin autoantibodies had a later manifestation of microalbuminuria at a mean of 10.1 years (95% CI 7.0 to 13.2 years). However, the observed mean difference of 2.4 years is statistically insignificant (*P*=0.42).

5.3.4 Immunofluorescence

Immunohistochemical stainings of rat kidney cortex sections were done using the three diabetic serum samples with highest levels of nephrin autoantibodies. This revealed an epithelial-type glomerular staining typical for nephrin with two of the sera. One patient serum sample showed negligible reactivity, as did the stainings with negative control sera and secondary antibodies alone (Study IV, Figure 2).

6 DISCUSSION

6.1 Nephrin and podocyte in proteinuric conditions

In studies I-III it was shown that under conditions of proteinuria and podocyte injury, the mRNA and protein expression levels of nephrin are altered. Our work was done in animals using the rat PAN and STZ models as well as NOD mice.

In our study with the PAN model, nephrin-alpha mRNA levels were shown to decrease. Puromycin aminonucleoside given as a single dose is toxic to the podocyte as reviewed earlier in chapter 2.4.1. Importantly, in the PAN model the podocytes and kidneys as a whole recover from the toxic insult (Ryan GB et al., 1975). Permanent podocyte loss doesn't seem to take place, while podocyte foot processes are temporarily detached (Messina A et al., 1987). This, and the *in vitro* data showing a decrease in the expression of α3β1-integrin in the podocytes (Krishnamurti U et al., 2001), suggests that the nephrin-alpha mRNA and nephrin mRNA level decreases seen in study I could at least partly be an actual sign of decreased production of the protein rather than a sign of massive podocyte death. In opposition to this interpretation are the *in vitro* results implicating also apoptosis in the detachment process (Suzuki T et al., 2001). However, podocytes are believed to be incapable of cell replication (Kriz W et al., 1998) and a massive podocyte loss in PAN model is not a typical feature seen in electron microscopic studies (Messina A et al., 1987). Accordingly, Suzuki and colleagues conclude in their article that apoptosis may only partly explain the detachment.

In studies II and III, kidney samples from experimental animals with long-standing proteinuria due to diabetic nephropathy showed a clear reduction in nephrin mRNA expression. This reduction in nephrin expression in association with long-standing proteinuria is confirmed by several other experimental studies (Forbes JM et al., 2002; Davis BJ et al., 2003; Cohen MP et al., 2005; Ichinose K et al., 2005). Nephrin mRNA expression is seen to decrease in human diabetic nephropathy as well (Langham RG et al., 2002; Toyoda M et al., 2004). However, the exact mechanisms behind this observed reduction in nephrin are not clear. In the kidney tissue, nephrin is specifically located in the podocytes. Therefore, nephrin also parallels the amount of podocytes in a given sample of kidney tissue. Since the podocytes can alter their gene expression levels of nephrin, as demonstrated by the PAN model used in study I, direct conclusions between nephrin mRNA amount and podocyte count in different samples and time points can be misleading. Nevertheless, in a condition with an abnormally permeable glomerular filtration system, it would seem contradictory that nephrin, a crucial component of the slit diaphragm, was physiologically downregulated. Instead, as was observed in study II, at the initial phases of diabetic nephropathy nephrin mRNA expression actually increased (Figure 5.1).

This was later confirmed by Forbes et al. (Forbes JM et al., 2002). Furthermore, the commonly acknowledged increase in PKC activity during diabetic nephropathy in the podocyte, as described in the literature review in chapter 2.5, seems to rather increase than decrease nephrin expression (Wang SX et al., 2001b). The genotoxic effects of STZ on the kidney should have been repaired already by the sample time points of 4 and 8 weeks in our study, as reviewed earlier in chapter 2.4. However, it is clear that preneoplastic or even neoplastic cells will prevail in the kidneys of STZ treated rats throughout their life, possibly causing some confounding results in longer-term studies using test compounds giving further tumor-promoting stimuli. As also shown by our results in study III, treatment with perindopril, an ACE inhibitor, appears to prevent the decrease of nephrin expression in STZ rats. Remarkably, another anti-proteinuric treatment with aminoguanidine, a drug reducing the formation of AGEs, did not prevent this drop in nephrin expression (Figure 5.1). Both treatments could reduce proteinuria, although it must be stressed, that the systolic blood pressure was clearly higher in the aminoguanidine –treated group as compared to the perindopril –treated group (Study III, Table 2). The adverse effect of high blood pressure was therefore an additional burden for the glomerular filtration barrier in the aminoguanidine treated STZ rats. In another study in STZ diabetic rats, the angiotensin II receptor type 1 antagonist valsartan conferred protection from reduced nephrin expression, while an equally hypotensive regimen with the calcium channel blocker amlodipine did not prevent the reduction (Davis BJ et al., 2003). The mechanism protecting from the decrease in nephrin mRNA expression levels appears to be related to RAS inhibition.

As is the case for STZ genotoxicity in kidney, it is unlikely that the absence of a delay before starting the treatment as suggested by Kraynak et al. (Kraynak AR et al., 1995) has affected our results of renoprotection in study III. First of all, in a very early sample at 1 week after STZ injection there was no change in nephrin mRNA expression (Figure 5.1), suggesting that podocytes or at least their nephrin expression is not significantly disturbed by DNA damage caused by STZ. Second, neither perindopril nor aminoguanidine are known to have significant tumor-promoting effects. On the contrary, perindopril is considered to be antiangiogenic and may even have potential for cancer therapies (Yoshiji H et al., 2002). In any case, these results suggest that treatment with RAS inhibitors may involve mechanisms in the podocyte which contribute to the survival of the podocyte as suggested by the retained normal expression levels of nephrin mRNA. Moreover, the ability of the podocyte to keep the filtration slit diaphragm functional seems to involve complex signalling in which nephrin is clearly implicated.

Nephrin-dependent signalling is also shown to be important for the podocyte to avoid apoptosis, induced e.g. by detachment of the podocyte from the GBM (Huber TB et al., 2003a). The lipid rafts that collect the important signalling proteins at the slit diaphragm can be disrupted during podocyte injury, leading to dislocation of these proteins to the apical part of the podocyte foot process cell membrane (Simons M et al., 2001). This loss of functional nephrin could lead to increased apoptosis due to loss of nephrin-signalling (see Figure 2.6.2). The podocyte could therefore be argued to be

forced to increase its expression of nephrin mRNA to compensate for this loss of functional nephrin.

Patients with type 1 diabetes show reduced counts of podocytes (Steffes MW et al., 2001) and in diabetic nephropathy podocytes appear to be shed into the urine. First, we detected nephrin protein in urine of diabetic rats by immunoblotting in study II. The approximate molecular weight of the urinary nephrin band blotted study II corresponds with the glomerular nephrin. Nephrin or fragments of nephrin are also detected in the urine of patients with type 1 diabetes (Pätäri A et al., 2003). Second, apical membrane fragments of podocytes with intact podocalyxin have been detected in the urine of patients with proteinuria (Hara M et al., 2005). Most convincingly, whole viable podocytes can be recovered from the urine in a model of diabetic nephropathy (Petermann AT et al., 2004). The evident loss of podocytes into the urine directly means also loss of nephrin protein on the membrane surfaces of these cells as well as loss of nephrin mRNA within their cytoplasm. Furthermore, the fact that podocytes are capable to avoid detachment-induced apoptosis in diabetic nephropathy shows that there must also be efficient anti-apoptotic signalling factors present. Nephrin-signalling is shown to be such a factor in the podocyte, and functional nephrin at the correct cell membrane domain is required for this signalling to take place (Huber TB et al., 2005). Podocyte survival would be compromised if nephrin expression in the podocyte was significantly reduced.

Taking all this together, a combined model for podocyte nephrin expression levels seen in diabetic nephropathy is here proposed. As mentioned above, the pathologic signalling changes and leaky glomerular filtration slit are suggested to increase rather than decrease the nephrin expression in the podocyte as a compensatory mechanism. At the initial stages of diabetic nephropathy the increased protein permeability of the filtration system is proposed to mainly be due to increased VEGF and its effects on the endothelium (Schrijvers BF et al., 2004). At this point, hyperglycaemic conditions increase PKC signalling in the podocytes, nephrin mRNA expression is seen to be increased, and in immunohistochemisty dislocation of nephrin protein expression to more central parts of the podocytes can be seen along with the overall increase in nephrin protein expression levels (Study II, Figure 7B).

Later, during the development of diabetic nephropathy, glomerular scarring and thickening of the GBM are histologically visible. At this stage, since scarring is seen, a significant number of podocytes must have been lost. The currently proposed interpretation of the results suggests that the nephrin mRNA decrease seen in many studies largely reflects the mass loss of podocytes. Expression of nephrin in single podocytes during diabetic nephropathy may be normal or even increased as at the initial stages of the pathologic process. Thus, a decrease in nephrin mRNA could be considered as a crude indicator of podocyte count in subjects with diabetic nephropathy. This can be argued because the methodological limitations of the PCR techniques must be taken into account. Genes used to normalize the expression levels between samples known to be expressed with constant levels in a given condition are called housekeeping genes. In the kidney the housekeeping genes used in the above mentioned expression

studies, including our own work, are expressed in virtually all cells present in the cortical kidney tissue. Since the total cell mass and, along with this, the total housekeeping gene expression in the kidneys is only minimally decreased due to podocyte loss, the determination of expression levels of a given gene specifically in a podocyte would require further research with application of single-cell PCR methods on isolated podocytes. Another approach to tackle this question could be to normalize the measured nephrin mRNA levels to some podocyte-specific housekeeping gene, i.e. a gene with known constant expression level. Currently, such gene is unfortunately not known. Also isolation of glomeruli can be used to enhance the accuracy of nephrin mRNA quantifications normalized with commonly expressed housekeeping gene mRNA levels. In line with the present interpretation, is has been shown that in various acquired kidney diseases nephrin protein levels visualized by immunohistochemistry are reduced whereas the glomerular mRNA levels of nephrin are increased (Koop K et al., 2003), possibly also representing an increased turn-over rate of nephrin protein product.

6.2 Immunological aspects of diabetic nephropathy

In study IV, circulating autoantibodies to nephrin were found in patients with type 1 diabetes. Glomeruli could also be stained in an epithelial-like pattern with patient sera of the highest nephrin autoantibody levels.

The first indicator of autoantibody generation in type 1 diabetes was the discovery of circulating antibodies against the islets of Langerhans (Bottazzo GF et al., 1974), now known to comprise antibodies to a heterogeneous set of islet cell antigens, such as insulin, glutamic acid decarboxylase (GAD) and IA-2 (Palmer JP et al., 1983; Baekkeskov S et al., 1990; Lan MS et al., 1996). The presence of these autoantibodies is associated with the early preclinical phase of type 1 diabetes. It is known that at least either one of GADA or IA-2A is present in more than 90% of patients with recent onset type 1 diabetes (Savola K et al., 1998). The nephrin autoantibody prevalence profile during the sampling period over the 10 first years of type 1 diabetes is similarly declining as the prevalence profile of the ICA, IA-2A or GADA. This suggests also that the nephrin autoantibodies are generated during the autoimmune process leading to type 1 diabetes. In any case, our result indicates that nephrin can act as an autoantigen in patients with type 1 diabetes. However, nephrin autoantibodies have a low prevalence at the time of diagnosis of clinical diabetes (Study IV, Table 2) and thus may not function as clinical markers of the disease process of diabetes itself.

Approximately one third of patients with type 1 diabetes develop diabetic nephropathy during the course of their disease (Rossing P et al., 1995). Some

patients experience a rapidly progressive glomerular injury, while others develop this complication only after decades. Accordingly, diabetic nephropathy seems to be a multifactorial disease. In 1993 it was for the first time shown that an intervention with ACE inhibitors could postpone diabetic nephropathy (Lewis EJ et al., 1993). However, it should be noted that even ACE inhibition is not capable in curing or completely preventing diabetic nephropathy. ACE inhibition is a routine treatment, although it is also associated with potential side effects. This emphasizes the importance of accurate early diagnostics to identify patients benefiting from intensive therapy. In an attempt to meet the needs of early diagnosis, microalbuminuria has been used as a marker for diabetic nephropathy (Mogensen CE et al., 1984). However, approximately 7% of nondiabetic, nonhypertensive subjects present with microalbuminuria as well (Tomura S et al., 1999; Hillege HL et al., 2001). Moreover, not all diabetic patients with microalbuminuria develop overt nephropathy and, furthermore, clear histopathological changes can develop without long-standing microalbuminuria (Chavers BM et al., 1989; Fioretto P et al., 1994). Taken together these facts call for novel early molecular markers of diabetic nephropathy (Caramori ML et al., 2000). To explore the potential of nephrin autoantibodies as such a marker we hypothesized that circulating nephrin autoantibodies partly explain diabetic damage of the glomerular filtration barrier. This hypothesis was founded on the facts that *in vivo* injected (Orikasa M et al., 1988; Topham PS et al., 1999) or naturally occurring antinephrin antibodies (Wang SX et al., 2001a; Patrakka J et al., 2002) cause glomerular damage and proteinuria.

Type 1 diabetic patients developing kidney complications, as indicated by the appearance of microalbuminuria or proteinuria, and those remaining unaffected present with statistically indifferent nephrin autoantibody proportions. During the follow-up period, only 4/29 (13.8%) patients positive for nephrin autoantibodies at any time point developed microalbuminuria or overt proteinuria. It is therefore unlikely that the nephrin autoantibodies significantly affect the development of diabetic nephropathy. Our test screened the samples mainly for autoantibodies against epitopes of the intracellular part of nephrin. In addition to covering one third of the extracellular part, we chose to cover the whole intracellular portion with our labeled nephrin probe. This was done, since the intracellular part is normally “hidden” from immune surveillance and immunotolerance. During the autoimmune process in the islets of Langerhans, nephrin autoantibodies were most likely to be generated by the process known as epitope spreading. The process of epitope spreading results from secondary endogenous priming with new self-antigens, after the primary generation of an immune response to disease-initiating self-antigens. It has been demonstrated that progression in autoimmune disease involves shifting of autoreactivity from primary initiating self-determinants to defined cascades of secondary determinants (Tuohy VK et al., 1999). Such epitope spreading occurs also in the autoimmune response to GAD during the preclinical stages of type 1 diabetes (Sohnlein P et al., 2000). Studies on T cell epitope spreading suggest that once the CD4+ T cell tolerance is broken for one epitope, the autoimmune response can

spread to other epitopes within the same autoantigen and even to nearby autoantigens (Lehmann PV et al., 1992; Miller SD et al., 1995). The autoimmune response in type 1 diabetes leads to beta cell damage, which may then lead to the exposure of hidden cytoplasmic epitopes rendering them available to an autoimmune attack. Due to these factors, to screen for nephrin autoantibodies in type 1 diabetes using mainly an intracellular epitope probe seemed to be the rational approach.

One explanation for the absence of significant damage to the kidneys could be that the intracellular nephrin epitopes are protected in the podocyte cytoplasm. Formation of linear deposits of IgG in the diabetic glomeruli has been reported earlier (Mauer SM et al., 1972; Axe SR et al., 1981). Although fluctuation in the individual nephrin autoantibody titers was seen in the present study, the subset of patients with constantly prevailing nephrin antibodies may increasingly accumulate these antibodies in the kidney. The pathogenicity of such IgG deposits in diabetic nephropathy remains unknown. It must also be stated, that the follow-up study was not originally designed to find out whether circulating autoantibodies could end up in the kidney glomerulus and attach to their respective epitopes. The immunohistochemical stainings with patient sera did not exclude this possibility, but the issue requires further study.

6.3 Conclusions

In conclusion, this thesis work could show changes of nephrin expression along with the development of proteinuria. In the PAN model, the nephrin-alpha and full-length nephrin expression levels appeared to decrease. In the initial stages of diabetic nephropathy, nephrin expression levels appear to increase. Later, in overt nephropathy, a decrease in nephrin mRNA expression could be seen, probably reflecting a declining amount of intact podocytes.

It was also shown that a subset of patients with type 1 diabetes have circulating autoantibodies to nephrin. These autoantibodies are most likely to be generated in the autoimmune process leading to type 1 diabetes. However, it is unlikely that the nephrin autoantibodies detected are significantly contributing to the development of diabetic nephropathy. Further research is needed to fully resolve the prognostic potential of such autoantibodies.

7 ACKNOWLEDGEMENTS

The work for my thesis was carried out at the University of Helsinki, at the Department of Bacteriology and Immunology in Haartman Institute and in Biomedicum Helsinki during the years 1999-2006. I would like to thank the current Head of the Department Professor Seppo Meri and his predecessor Risto Renkonen for providing the excellent facilities to conduct my work.

Particularly grateful I am to Docent Harry Holthöfer, my supervisor. His inspirative attitude towards science attracted me to the world of research in the first place. He has always been understanding and supportive.

The most helpful comments and corrections made by the reviewers of this thesis, Professor Ilkka Pörsti and Docent Tiinamaija Tuomi, are appreciatively recognized.

I am also grateful to the fellow research scientists in our laboratory, as well as to all the other co-authors of the original publications. Thanks to all the past and present members of our research group, particularly: Johanna Rinta-Valkama, Pauliina Luimula, Heikki Ahola, Tuula Palmén, Anu Pätäri, Eija Heikkilä, Marika Havana, Pekka Ihalmo, Shixuan Wang, Zhu-Zhu Cheng, Marcel Messing, Eva Åström, Harri Tapanainen, Juuso Juhila and Liisa Pirinen, I have learned so much from you!

I would like to thank all my friends and family members for providing me with a contact to the life outside the laboratory. I want to specially thank my father Pertti for showing me the example of a serious scientist succeeding also in other sectors of life.

My thesis work would not have been possible without the financial support from the Finnish Medical Foundation, the Paulo Foundation, the Finnish Cultural Foundation and the University of Helsinki.

Most importantly, I would like to thank my wife Gisele for her loving support. Especially during the last few months of hard work you gave me the strength to carry on writing. Thank you.

Helsinki, April 2006

Petri Aaltonen

8 REFERENCES

Abbas AK, Lohr J, Knoechel B, Nagabhushanam V. T cell tolerance and autoimmunity. Autoimmun Rev vol. 3, p. 471-5, 2004.

Abraham RS, Kudva YC, Wilson SB, Strominger JL, David CS. Co-expression of HLA DR3 and DQ8 results in the development of spontaneous insulitis and loss of tolerance to GAD65 in transgenic mice. Diabetes vol. 49, p. 548-54, 2000.

Ahola H, Heikkila E, Astrom E, Inagaki M, Izawa I, Pavenstadt H, Kerjaschki D, Holthofer H. A novel protein, densin, expressed by glomerular podocytes. J Am Soc Nephrol vol. 14, p. 1731-7, 2003.

Ahola H, Wang SX, Luimula P, Solin ML, Holzman LB, Holthofer H. Cloning and expression of the rat nephrin homolog. Am J Pathol vol. 155, p. 907-13, 1999.

Anjos S, Polychronakos C. Mechanisms of genetic susceptibility to type I diabetes: beyond HLA. Mol Genet Metab vol. 81, p. 187-95, 2004.

Aoki CA, Borchers AT, Ridgway WM, Keen CL, Ansari AA, Gershwin ME. NOD mice and autoimmunity. Autoimmun Rev vol. 4, p. 373-9, 2005.

Apperson ML, Moon IS, Kennedy MB. Characterization of densin-180, a new brain-specific synaptic protein of the O-sialoglycoprotein family. J Neurosci vol. 16, p. 6839-52, 1996.

Araki S, Ng DP, Krolewski B, Wyrwicz L, Rogus JJ, Canani L, Makita Y, Haneda M, Warram JH, Krolewski AS. Identification of a common risk haplotype for diabetic nephropathy at the protein kinase C-beta1 (PRKCB1) gene locus. J Am Soc Nephrol vol. 14, p. 2015-24, 2003.

Arvan P, Halban PA. Sorting ourselves out: seeking consensus on trafficking in the beta-cell. Traffic vol. 5, p. 53-61, 2004.

Atchley DH, Lopes-Virella MF, Zheng D, Kenny D, Virella G. Oxidized LDL-anti-oxidized LDL immune complexes and diabetic nephropathy. Diabetologia vol. 45, p. 1562-71, 2002.

Axe SR, Katz SM, Lavine R. Immunoglobulin deposition in the microvasculature of the streptozotocin-induced diabetic rat. Lab Invest vol. 45, p. 229-33, 1981.

Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, De Camilli P, Camilli PD. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature vol. 347, p. 151-6, 1990.

Barletta GM, Kovari IA, Verma RK, Kerjaschki D, Holzman LB. Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form cis hetero-oligomers. J Biol Chem vol. 278, p. 19266-71, 2003.

Barlow SC, Langston W, Matthews KM, Chidlow JH,Jr, Kevil CG. CD18 deficiency protects against multiple low-dose streptozotocin-induced diabetes. Am J Pathol vol. 165, p. 1849-52, 2004.

Beall MH, Amidi F, Gayle DA, Wang S, Beloosesky R, Ross MG. Placental and fetal membrane Nephrin and Neph1 gene expression: response to inflammation. J Soc Gynecol Investig vol. 12, p. 298-302, 2005.

Bell GI, Horita S, Karam JH. A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. Diabetes vol. 33, p. 176-83, 1984.

Bennett RA, Pegg AE. Alkylation of DNA in rat tissues following administration of streptozotocin. Cancer Res vol. 41, p. 2786-90, 1981.

Bennett ST, Lucassen AM, Gough SC, Powell EE, Undlien DE, Pritchard LE, Merriman ME, Kawaguchi Y, Dronsfield MJ, Pociot F. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. Nat Genet vol. 9, p. 284-92, 1995.

Bilder D, Birnbaum D, Borg JP, Bryant P, Huigbretse J, Jansen E, Kennedy MB, Labouesse M, Legouis R, Mechler B, Perrimon N, Petit M, Sinha P. Collective nomenclature for LAP proteins. Nat Cell Biol vol. 2, p. E114, 2000.

Bojestig M, Arnqvist HJ, Hermansson G, Karlberg BE, Ludvigsson J. Declining incidence of nephropathy in insulin-dependent diabetes mellitus. N Engl J Med vol. 330, p. 15-8, 1994.

Bolzan AD, Bianchi MS. Genotoxicity of streptozotocin. Mutat Res vol. 512, p. 121-34, 2002.

Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG, Gamble DR. In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. N Engl J Med vol. 313, p. 353-60, 1985.

Bottazzo GF, Florin-Christensen A, Doniach D. Islet-cell antibodies in diabetes mellitus with autoimmune

polyendocrine deficiencies. Lancet vol. 2, p. 1279-83, 1974.

Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K, Rostamkhani M, MacMurray J, Meloni GF, Lucarelli P, Pellecchia M, Eisenbarth GS, Comings D, Mustelin T. A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nat Genet vol. 36, p. 337-8, 2004.

Boukhelifa M, Parast MM, Bear JE, Gertler FB, Otey CA. Palladin is a novel binding partner for Ena/VASP family members. Cell Motil Cytoskeleton vol. 58, p. 17-29, 2004.

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

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

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

Buschard K, Josefsen K, Horn T, Larsen S, Fredman P. Sulphatide antigen in islets of Langerhans and in diabetic glomeruli, and anti-sulphatide antibodies in type 1 diabetes mellitus. APMIS vol. 101, p. 963-70, 1993.

Caramori ML, Fioretto P, Mauer M. The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient? Diabetes vol. 49, p. 1399-408, 2000.

Caulfield JP, Reid JJ, Farquhar MG. Alterations of the glomerular epithelium in acute aminonucleoside nephrosis. Evidence for formation of occluding junctions and epithelial cell detachment. Lab Invest vol. 34, p. 43-59, 1976.

Chang PY, Benecke H, Le Marchand-Brustel Y, Lawitts J, Moller DE. Expression of a dominant-negative mutant human insulin receptor in the muscle of transgenic mice. J Biol Chem vol. 269, p. 16034-40, 1994.

Charonis AS, Reger LA, Dege JE, Kouzi-Koliakos K, Furcht LT, Wohlhueter RM, Tsilibary EC. Laminin alterations after in vitro nonenzymatic glycosylation. Diabetes vol. 39, p. 807-14, 1990.

Chavers BM, Bilous RW, Ellis EN, Steffes MW, Mauer SM. Glomerular lesions and urinary albumin excretion in type I diabetes without overt proteinuria. N Engl J Med vol. 320, p. 966-70, 1989.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem vol. 162, p. 156-9, 1987.

Cohen MP, Chen S, Ziyadeh FN, Shea E, Hud EA, Lautenslager GT, Shearman CW. Evidence linking glycated albumin to altered glomerular nephrin and VEGF expression, proteinuria, and diabetic nephropathy. Kidney Int vol. 68, p. 1554-61, 2005.

Conaway HH, Faas FH, Smith SD, Sanders LL. Spontaneous diabetes mellitus in the New Zealand white rabbit: physiologic characteristics. Metabolism vol. 30, p. 50-6, 1981.

Coward RJ, Welsh GI, Koziell A, Tavare JM, Mathieson PW, Saleem MA. In: Nephrin is critical for the action of insulin on human podocytes. American Society of Nephrology Abstracts: F-PO475, 2005a.

Coward RJ, Welsh GI, Yang J, Tasman C, Lennon R, Koziell A, Satchell S, Holman GD, Kerjaschki D, Tavare JM, Mathieson PW, Saleem MA. The Human Glomerular Podocyte Is a Novel Target for Insulin Action. Diabetes vol. 54, p. 3095-102, 2005b.

Dahlquist G, Blom L, Tuvemo T, Nystrom L, Sandstrom A, Wall S. The Swedish childhood diabetes study--results from a nine year case register and a one year case-referent study indicating that type 1 (insulin-dependent) diabetes mellitus is associated with both type 2 (non-insulin-dependent) diabetes mellitus and autoimmune disorders. Diabetologia vol. 32, p. 2-6, 1989.

Davis BJ, Cao Z, de Gasparo M, Kawachi H, Cooper ME, Allen TJ. Disparate effects of angiotensin II antagonists and calcium channel blockers on albuminuria in experimental diabetes and hypertension: potential role of nephrin. J Hypertens vol. 21, p. 209-16, 2003.

Degenhardt TP, Thorpe SR, Baynes JW. Chemical modification of proteins by methylglyoxal. Cell Mol Biol (Noisy-le-grand) vol. 44, p. 1139-45, 1998.

Di Paolo S, Gesualdo L, Ranieri E, Grandaliano G, Schena FP. High glucose concentration induces the overexpression of transforming growth factor-beta through the activation of a platelet-derived growth factor loop in human mesangial cells. Am J Pathol vol. 149, p. 2095-106, 1996.

Ditzel J, Schwartz M. Abnormally increased glomerular filtration rate in short-term insulin-treated diabetic subjects. Diabetes vol. 16, p. 264-7, 1967.

Donoviel DB, Freed DD, Vogel H, Potter DG, Hawkins E, Barrish JP, Mathur BN, Turner CA, Geske R, Montgomery CA, Starbuck M, Brandt M, Gupta A, Ramirez-Solis R, Zambrowicz BP, Powell DR. Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. Mol Cell Biol vol. 21, p. 4829-36, 2001.

Doyonnas R, Kershaw DB, Duhme C, Merkens H, Chelliah S, Graf T, McNagny KM. Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin. J Exp Med vol. 194, p. 13-27, 2001.

Drenckhahn D, Franke RP. Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man. Lab Invest vol. 59, p. 673-82, 1988.

Durbeej M, Campbell KP. Biochemical characterization of the epithelial dystroglycan complex. J Biol Chem vol. 274, p. 26609-16, 1999.

Dustin ML, Olszowy MW, Holdorf AD, Li J, Bromley S, Desai N, Widder P, Rosenberger F, van der Merwe PA, Allen PM, Shaw AS. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. Cell vol. 94, p. 667-77, 1998.

Eskridge EM, Shields D. The NH2 terminus of preproinsulin directs the translocation and glycosylation of a bacterial cytoplasmic protein by mammalian microsomal membranes. J Cell Biol vol. 103, p. 2263-72, 1986.

Esmatjes E, De Alvaro F, 'Estudio Diamante' Investigators. Incidence of diabetic nephropathy in Type 1 diabetic patients in Spain: 'Estudio Diamante'. Diabetes Res Clin Pract vol. 57, p. 35-43, 2002.

Fagerudd JA, Pettersson-Fernholm KJ, Gronhagen-Riska C, Groop PH. The impact of a family history of Type II (non-insulin-dependent) diabetes mellitus on the risk of diabetic nephropathy in patients with Type I (insulin-dependent) diabetes mellitus. Diabetologia vol. 42, p. 519-26, 1999.

Feld LG, Cachero S, Ellis EN, Dedeoglu O, Ueda Y, Leonard C, Letizia R, Mallon V, Van Liew JB. Resistance to glomerular injury in the diabetic biobreeding rat. Exp Physiol vol. 80, p. 991-1000, 1995.

Finnish Registry for Kidney Diseases. Report 2004. Annual Data Report. Finland, 2005.

Fioretto P, Steffes MW, Mauer M. Glomerular structure in nonproteinuric IDDM patients with various levels of albuminuria. Diabetes vol. 43, p. 1358-64, 1994.

Forbes JM, Bonnet F, Russo LM, Burns WC, Cao Z, Candido R, Kawachi H, Allen TJ, Cooper ME, Jerums G, Osicka TM. Modulation of nephrin in the diabetic kidney: association with systemic hypertension and increasing albuminuria. J Hypertens vol. 20, p. 985-92, 2002.

Freedman BI, Tuttle AB, Spray BJ. Familial predisposition to nephropathy in African-Americans with non-insulin-dependent diabetes mellitus. Am J Kidney Dis vol. 25, p. 710-3, 1995.

Ganong WF. Endocrine Functions of the Kidneys, Heart, & Pineal Gland. In: Review of Medical Physiology. 21st ed. McGraw-Hill, p. 458-63, 2003a.

Ganong WF. Renal Function & Micturition. In: Review of Medical Physiology. 21st ed. McGraw-Hill, p. 702-32, 2003b.

Ganong WF. Endocrine Functions of the Pancreas & Regulation of Carbohydrate Metabolism. In: Review of Medical Physiology. 21st ed. McGraw-Hill, p. 336-58, 2003c.

Gartner LP, Hiatt JL. Urinary System. In: Color textbook of histology. 1st ed. W.B. Saunders Company, p. 358-81, 1997a.

Gartner LP, Hiatt JL. Digestive System III - Glands. In: Color textbook of histology. 1st ed. W.B. Saunders Company, p. 341-6, 1997b.

Gerke P, Sellin L, Kretz O, Petraschka D, Zentgraf H, Benzing T, Walz G. NEPH2 is located at the glomerular slit diaphragm, interacts with nephrin and is cleaved from podocytes by metalloproteinases. J Am Soc Nephrol vol. 16, p. 1693-702, 2005.

Gilbert RE, Rumble JR, Cao Z, Cox AJ, van Eeden P, Allen TJ, Kelly DJ, Cooper ME. Endothelin receptor antagonism ameliorates mast cell infiltration, vascular hypertrophy, and epidermal growth factor expression in experimental diabetes. Circ Res vol. 86, p. 158-65, 2000.

Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. Diabetes Care vol. 19, p. 257-67, 1996.

Gnudi L, Viberti G, Raij L, Rodriguez V, Burt D, Cortes P, Hartley B, Thomas S, Maestrini S, Gruden G. GLUT-1 overexpression: Link between hemodynamic and metabolic factors in glomerular injury? Hypertension vol. 42, p. 19-24, 2003.

Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, Karlsen AE, Boel E, Michelsen B, Lernmark A. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia vol. 37, p. 344-50, 1994.

Gruden G, Thomas S, Burt D, Lane S, Chusney G, Sacks S, Viberti G. Mechanical stretch induces vascular

permeability factor in human mesangial cells: mechanisms of signal transduction. Proc Natl Acad Sci U S A vol. 94, p. 12112-6, 1997.

Grupe A, Hultgren B, Ryan A, Ma YH, Bauer M, Stewart TA. Transgenic knockouts reveal a critical requirement for pancreatic beta cell glucokinase in maintaining glucose homeostasis. Cell vol. 83, p. 69-78, 1995.

Guberski DL, Thomas VA, Shek WR, Like AA, Handler ES, Rossini AA, Wallace JE, Welsh RM. Induction of type I diabetes by Kilham's rat virus in diabetes-resistant BB/Wor rats. Science vol. 254, p. 1010-3, 1991.

Hara M, Yanagihara T, Kihara I, Higashi K, Fujimoto K, Kajita T. Apical cell membranes are shed into urine from injured podocytes: a novel phenomenon of podocyte injury. J Am Soc Nephrol vol. 16, p. 408-16, 2005.

Harita Y, Miyauchi N, Karasawa T, Suzuki K, Han GD, Koike H, Igarashi T, Shimizu F, Kawachi H. Altered expression of junctional adhesion molecule 4 (JAM4) in injured podocytes. Am J Physiol Renal Physiol 2005.

Harjutsalo V, Podar T, Tuomilehto J. Cumulative incidence of type 1 diabetes in 10,168 siblings of Finnish young-onset type 1 diabetic patients. Diabetes vol. 54, p. 563-9, 2005.

Harper ME, Ullrich A, Saunders GF. Localization of the human insulin gene to the distal end of the short arm of chromosome 11. Proc Natl Acad Sci U S A vol. 78, p. 4458-60, 1981.

Hillege HL, Janssen WM, Bak AA, Diercks GF, Grobbee DE, Crijns HJ, Van Gilst WH, De Zeeuw D, De Jong PE, Prevend Study Group. Microalbuminuria is common, also in a nondiabetic, nonhypertensive population, and an independent indicator of cardiovascular risk factors and cardiovascular morbidity. J Intern Med vol. 249, p. 519-26, 2001.

Hirabayashi S, Mori H, Kansaku A, Kurihara H, Sakai T, Shimizu F, Kawachi H, Hata Y. MAGI-1 is a component of the glomerular slit diaphragm that is tightly associated with nephrin. Lab Invest vol. 85, p. 1528-43, 2005.

Hjalmarsson C, Johansson BR, Haraldsson B. Electron microscopic evaluation of the endothelial surface layer of glomerular capillaries. Microvasc Res vol. 67, p. 9-17, 2004.

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

Holthofer H, Kretzler M, Haltia A, Solin ML, Taanman JW, Schagger H, Kriz W, Kerjaschki D, Schlondorff D. Altered gene expression and functions of mitochondria in human nephrotic syndrome. FASEB J vol. 13, p. 523-32, 1999b.

Horster M, Huber S, Tschop J, Dittrich G, Braun G. Epithelial nephrogenesis. Pflugers Arch vol. 434, p. 647-60, 1997.

Hoshi S, Nomoto K, Kuromitsu J, Tomari S, Nagata M. High glucose induced VEGF expression via PKC and ERK in glomerular podocytes. Biochem Biophys Res Commun vol. 290, p. 177-84, 2002.

Hostetter TH. Diabetic Nephropathy. In: Brenner BM, Rector FC, editors. The Kidney. 4th ed. W.B. Saunders Company, 1991.

Hovind P, Tarnow L, Rossing P, Jensen BR, Graae M, Torp I, Binder C, Parving HH. Predictors for the development of microalbuminuria and macroalbuminuria in patients with type 1 diabetes: inception cohort study. BMJ vol. 328, p. 1105, 2004.

Hovind P, Tarnow L, Rossing K, Rossing P, Eising S, Larsen N, Binder C, Parving HH. Decreasing incidence of severe diabetic microangiopathy in type 1 diabetes. Diabetes Care vol. 26, p. 1258-64, 2003.

Huber TB, Benzing T. The slit diaphragm: a signaling platform to regulate podocyte function. Curr Opin Nephrol Hypertens vol. 14, p. 211-6, 2005.

Huber TB, Hartleben B, Kim J, Schmidts M, Schermer B, Keil A, Egger L, Lecha RL, Borner C, Pavenstadt H, Shaw AS, Walz G, Benzing T. Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. Mol Cell Biol vol. 23, p. 4917-28, 2003a.

Huber TB, Schmidts M, Gerke P, Schermer B, Zahn A, Hartleben B, Sellin L, Walz G, Benzing T. The carboxyl terminus of Neph family members binds to the PDZ domain protein zonula occludens-1. J Biol Chem vol. 278, p. 13417-21, 2003b.

Huber TB, Kottgen M, Schilling B, Walz G, Benzing T. Interaction with podocin facilitates nephrin signaling. J Biol Chem vol. 276, p. 41543-6, 2001.

Hughson M, Farris AB,3rd, Douglas-Denton R, Hoy WE, Bertram JF. Glomerular number and size in autopsy kidneys: the relationship to birth weight. Kidney Int vol. 63, p. 2113-22, 2003.

Hyttinen V, Kaprio J, Kinnunen L, Koskenvuo M, Tuomilehto J. Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs: a nationwide follow-up study. Diabetes vol. 52, p. 1052-5, 2003.

Ichinose K, Maeshima Y, Yamamoto Y, Kitayama H, Takazawa Y, Hirokoshi K, Sugiyama H, Yamasaki Y, Eguchi K, Makino H. Antiangiogenic endostatin peptide ameliorates renal alterations in the early stage of a type 1 diabetic nephropathy model. Diabetes vol. 54, p. 2891-903, 2005.

Ihalmo P, Palmen T, Ahola H, Valtonen E, Holthofer H. Filtrin is a novel member of nephrin-like proteins. Biochem Biophys Res Commun vol. 300, p. 364-70, 2003.

Ilonen J, Sjoroos M, Knip M, Veijola R, Simell O, Akerblom HK, Paschou P, Bozas E, Havarani B, Malamitsi-Puchner A, Thymelli J, Vazeou A, Bartsocas CS. Estimation of genetic risk for type 1 diabetes. Am J Med Genet vol. 115, p. 30-6, 2002.

Inoki K, Haneda M, Maeda S, Koya D, Kikkawa R. TGF-beta 1 stimulates glucose uptake by enhancing GLUT1 expression in mesangial cells. Kidney Int vol. 55, p. 1704-12, 1999.

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

Iyengar SK, Fox KA, Schachere M, Manzoor F, Slaughter ME, Covic AM, Orloff SM, Hayden PS, Olson JM, Schelling JR, Sedor JR. Linkage analysis of candidate loci for end-stage renal disease due to diabetic nephropathy. J Am Soc Nephrol vol. 14, p. S195-201, 2003.

Johansson KA, Grapin-Botton A. Development and diseases of the pancreas. Clin Genet vol. 62, p. 14-23, 2002.

Joshi RL, Lamothe B, Cordonnier N, Mesbah K, Monthioux E, Jami J, Bucchini D. Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. EMBO J vol. 15, p. 1542-7, 1996.

Junod A, Lambert AE, Orci L, Pictet R, Gonet AE, Renold AE. Studies of the diabetogenic action of streptozotocin. Proc Soc Exp Biol Med vol. 126, p. 201-5, 1967.

Karle SM, Uetz B, Ronner V, Glaeser L, Hildebrandt F, Fuchshuber A. Novel mutations in NPHS2 detected in both familial and sporadic steroid-resistant nephrotic syndrome. J Am Soc Nephrol vol. 13, p. 388-93, 2002.

Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care vol. 23, p. 1516-26, 2000.

Karvonen M, Pitkaniemi J, Tuomilehto J. The onset age of type 1 diabetes in Finnish children has become younger. The Finnish Childhood Diabetes Registry Group. Diabetes Care vol. 22, p. 1066-70, 1999.

Kavvoura FK, Ioannidis JP. CTLA-4 gene polymorphisms and susceptibility to type 1 diabetes mellitus: a HuGE Review and meta-analysis. Am J Epidemiol vol. 162, p. 3-16, 2005.

Kawachi H, Kurihara H, Topham PS, Brown D, Shia MA, Orikasa M, Shimizu F, Salant DJ. Slit diaphragm-reactive nephritogenic MAb 5-1-6 alters expression of ZO-1 in rat podocytes. Am J Physiol vol. 273, p. F984-93, 1997.

Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, Natori T. New inbred strain of Long-Evans Tokushima lean rats with IDDM without lymphopenia. Diabetes vol. 40, p. 1375-81, 1991.

Kelly MA, Rayner ML, Mijovic CH, Barnett AH. Molecular aspects of type 1 diabetes. Mol Pathol vol. 56, p. 1-10, 2003.

Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. J Clin Invest vol. 108, p. 1583-7, 2001.

Kerjaschki D, Exner M, Ullrich R, Susani M, Curtiss LK, Witztum JL, Farquhar MG, Orlando RA. Pathogenic antibodies inhibit the binding of apolipoproteins to megalin/gp330 in passive Heymann nephritis. J Clin Invest vol. 100, p. 2303-9, 1997.

Kerjaschki D, Sharkey DJ, Farquhar MG. Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell. J Cell Biol vol. 98, p. 1591-6, 1984.

Kerjaschki D, Farquhar MG. Immunocytochemical localization of the Heymann nephritis antigen (GP330) in glomerular epithelial cells of normal Lewis rats. J Exp Med vol. 157, p. 667-86, 1983.

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

Khoshnoodi J, Sigmundsson K, Ofverstedt LG, Skoglund U, Obrink B, Wartiovaara J, Tryggvason K. Nephrin promotes cell-cell adhesion through homophilic interactions. Am J Pathol vol. 163, p. 2337-46, 2003.

Kim W, Hudson BI, Moser B, Guo J, Rong LL, Lu Y, Qu W, Lalla E, Lerner S, Chen Y, Yan SS, D'Agati V, Naka Y, Ramasamy R, Herold K, Yan SF, Schmidt

AM. Receptor for advanced glycation end products and its ligands: a journey from the complications of diabetes to its pathogenesis. Ann N Y Acad Sci vol. 1043, p. 553-61, 2005.

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

Kintzel PE. Anticancer drug-induced kidney disorders. Drug Saf vol. 24, p. 19-38, 2001.

Knip M, Veijola R, Virtanen SM, Hyoty H, Vaarala O, Akerblom HK. Environmental triggers and determinants of type 1 diabetes. Diabetes vol. 54 Suppl 2, p. S125-36, 2005.

Komeda K, Noda M, Terao K, Kuzuya N, Kanazawa M, Kanazawa Y. Establishment of two substrains, diabetes-prone and non-diabetic, from Long-Evans Tokushima Lean (LETL) rats. Endocr J vol. 45, p. 737-44, 1998.

Koop K, Eikmans M, Baelde HJ, Kawachi H, De Heer E, Paul LC, Bruijn JA. Expression of podocyte-associated molecules in acquired human kidney diseases. J Am Soc Nephrol vol. 14, p. 2063-71, 2003.

Korpinen E, Groop PH, Akerblom HK, Vaarala O. Immune response to glycated and oxidized LDL in IDDM patients with and without renal disease. Diabetes Care vol. 20, p. 1168-71, 1997.

Koya D, King GL. Protein kinase C activation and the development of diabetic complications. Diabetes vol. 47, p. 859-66, 1998.

Koziell A, Grech V, Hussain S, Lee G, Lenkkeri U, Tryggvason K, Scambler P. Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. Hum Mol Genet vol. 11, p. 379-88, 2002.

Kramer JW, Nottingham S, Robinette J, Lenz G, Sylvester S, Dessouky MI. Inherited, early onset, insulin-requiring diabetes mellitus of Keeshond dogs. Diabetes vol. 29, p. 558-65, 1980.

Kraynak AR, Storer RD, Jensen RD, Kloss MW, Soper KA, Clair JH, DeLuca JG, Nichols WW, Eydelloth RS. Extent and persistence of streptozotocin-induced DNA damage and cell proliferation in rat kidney as determined by in vivo alkaline elution and BrdUrd labeling assays. Toxicol Appl Pharmacol vol. 135, p. 279-86, 1995.

Krishnamurti U, Zhou B, Fan WW, Tsilibary E, Wayner E, Kim Y, Kashtan CE, Michael A. Puromycin aminonucleoside suppresses integrin expression in cultured glomerular epithelial cells. J Am Soc Nephrol vol. 12, p. 758-66, 2001.

Kriz W, Gretz N, Lemley KV. Progression of glomerular diseases: is the podocyte the culprit? Kidney Int vol. 54, p. 687-97, 1998.

Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H, Satoh S, Sekihara H, Sciacchitano S, Lesniak M, Aizawa S, Nagai R, Kimura S, Akanuma Y, Taylor SI, Kadowaki T. Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. Diabetes vol. 49, p. 1880-9, 2000.

Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR. Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell vol. 96, p. 329-39, 1999.

Kumar V, Cotran RS, Robbins SL, editors. Basic Pathology. 5th ed. W.B. Saunders Company, 1992.

Kuusniemi AM, Kestila M, Patrakka J, Lahdenkari AT, Ruotsalainen V, Holmberg C, Karikoski R, Salonen R, Tryggvason K, Jalanko H. Tissue expression of nephrin in human and pig. Pediatr Res vol. 55, p. 774-81, 2004.

Kyvik KO, Green A, Beck-Nielsen H. Concordance rates of insulin dependent diabetes mellitus: a population based study of young Danish twins. BMJ vol. 311, p. 913-7, 1995.

Ladner MB, Bottini N, Valdes AM, Noble JA. Association of the single nucleotide polymorphism C1858T of the PTPN22 gene with type 1 diabetes. Hum Immunol vol. 66, p. 60-4, 2005.

Lahdenpera J, Kilpelainen P, Liu XL, Pikkarainen T, Reponen P, Ruotsalainen V, Tryggvason K. Clustering-induced tyrosine phosphorylation of nephrin by Src family kinases. Kidney Int vol. 64, p. 404-13, 2003.

Lan MS, Wasserfall C, Maclaren NK, Notkins AL. IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. Proc Natl Acad Sci U S A vol. 93, p. 6367-70, 1996.

Lan RY, Ansari AA, Lian ZX, Gershwin ME. Regulatory T cells: development, function and role in autoimmunity. Autoimmun Rev vol. 4, p. 351-63, 2005.

Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. J Biol Chem vol. 272, p. 17810-4, 1997.

Langham RG, Kelly DJ, Cox AJ, Thomson NM, Holthofer H, Zaoui P, Pinel N, Cordonnier DJ, Gilbert RE. Proteinuria and the expression of the podocyte slit diaphragm protein, nephrin, in diabetic nephropathy: effects of angiotensin converting enzyme inhibition. Diabetologia vol. 45, p. 1572-6, 2002.

Laurendeau I, Bahuau M, Vodovar N, Larramendy C, Olivi M, Bieche I, Vidaud M, Vidaud D. TaqMan PCR-based gene dosage assay for predictive testing in individuals from a cancer family with INK4 locus haploinsufficiency. Clin Chem vol. 45, p. 982-6, 1999.

Leheste JR, Melsen F, Wellner M, Jansen P, Schlichting U, Renner-Muller I, Andreassen TT, Wolf E, Bachmann S, Nykjaer A, Willnow TE. Hypocalcemia and osteopathy in mice with kidney-specific megalin gene defect. FASEB J vol. 17, p. 247-9, 2003.

Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature vol. 358, p. 155-7, 1992.

Lehtonen S, Ryan JJ, Kudlicka K, Iino N, Zhou H, Farquhar MG. Cell junction-associated proteins IQGAP1, MAGI-2, CASK, spectrins, and alpha-actinin are components of the nephrin multiprotein complex. Proc Natl Acad Sci U S A vol. 102, p. 9814-9, 2005.

Lehtonen S, Lehtonen E, Kudlicka K, Holthofer H, Farquhar MG. Nephrin forms a complex with adherens junction proteins and CASK in podocytes and in Madin-Darby canine kidney cells expressing nephrin. Am J Pathol vol. 165, p. 923-36, 2004.

Leinonen JS, Rantalaiho V, Laippala P, Wirta O, Pasternack A, Alho H, Jaakkola O, Yla-Herttuala S, Koivula T, Lehtimaki T. The level of autoantibodies against oxidized LDL is not associated with the presence of coronary heart disease or diabetic kidney disease in patients with non-insulin-dependent diabetes mellitus. Free Radic Res vol. 29, p. 137-41, 1998.

Lenkkeri U, Mannikko M, McCready P, Lamerdin J, Gribouval O, Niaudet PM, Antignac C K, Kashtan CE, Homberg C, Olsen A, Kestila M, Tryggvason K. Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations. Am J Hum Genet vol. 64, p. 51-61, 1999.

Lewis C, Barbiers AR. Streptozotocin, a new antibiotic. In vitro and in vivo evaluation. Antibiot Annu vol. 7, p. 247-54, 1959.

Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. N Engl J Med vol. 329, p. 1456-62, 1993.

Li H, Lemay S, Aoudjit L, Kawachi H, Takano T. SRC-family kinase Fyn phosphorylates the cytoplasmic domain of nephrin and modulates its interaction with podocin. J Am Soc Nephrol vol. 15, p. 3006-15, 2004.

Li H, Lindholm E, Almgren P, Gustafsson A, Forsblom C, Groop L, Tuomi T. Possible human leukocyte antigen-mediated genetic interaction between type 1 and type 2 Diabetes. J Clin Endocrinol Metab vol. 86, p. 574-82, 2001.

Lindner TH, Monks D, Wanner C, Berger M. Genetic aspects of diabetic nephropathy. Kidney Int Suppl vol. 84, p. S186-91, 2003.

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

Liu ML, Gibbs EM, McCoid SC, Milici AJ, Stukenbrok HA, McPherson RK, Treadway JL, Pessin JE. Transgenic mice expressing the human GLUT4/muscle-fat facilitative glucose transporter protein exhibit efficient glycemic control. Proc Natl Acad Sci U S A vol. 90, p. 11346-50, 1993.

Lomedico P, Rosenthal N, Efstratidadis A, Gilbert W, Kolodner R, Tizard R. The structure and evolution of the two nonallelic rat preproinsulin genes. Cell vol. 18, p. 545-58, 1979.

Lopes-Virella MF, Virella G. Cytokines, modified lipoproteins, and arteriosclerosis in diabetes. Diabetes vol. 45 Suppl 3, p. S40-4, 1996.

Luimula P, Sandstrom N, Novikov D, Holthofer H. Podocyte-associated molecules in puromycin aminonucleoside nephrosis of the rat. Lab Invest vol. 82, p. 713-8, 2002.

Luimula P, Ahola H, Wang SX, Solin ML, Aaltonen P, Tikkanen I, Kerjaschki D, Holthofer H. Nephrin in experimental glomerular disease. Kidney Int vol. 58, p. 1461-8, 2000.

Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu vol. 29, p. 1-13, 1980.

Marsh SG, Albert ED, Bodmer WF, Bontrop RE, Dupont B, Erlich HA, Geraghty DE, Hansen JA, Mach B, Mayr WR, Parham P, Petersdorf EW, Sasazuki T, Schreuder GM, Strominger JL, Svejgaard A, Terasaki PI. Nomenclature for factors of the HLA system, 2002. Tissue Antigens vol. 60, p. 407-64, 2002.

Matsui K, Breiteneder-Geleff S, Kerjaschki D. Epitope-specific antibodies to the 43-kD glomerular membrane protein podoplanin cause proteinuria and rapid flattening of podocytes. J Am Soc Nephrol vol. 9, p. 2013-26, 1998.

Mauer SM, Michael AF, Fish AJ, Brown DM. Spontaneous immunoglobulin and complement deposition in glomeruli of diabetic rats. Lab Invest vol. 27, p. 488-94, 1972.

Merta M, Reiterova J, Rysava R, Kmentova D, Tesar V. Genetics of diabetic nephropathy. Nephrol Dial Transplant vol. 18 Suppl 5, p. v24-5, 2003.

Messina A, Davies DJ, Dillane PC, Ryan GB. Glomerular epithelial abnormalities associated with the onset of proteinuria in aminonucleoside nephrosis. Am J Pathol vol. 126, p. 220-9, 1987.

Miller SD, McRae BL, Vanderlugt CL, Nikcevich KM, Pope JG, Pope L, Karpus WJ. Evolution of the T-cell repertoire during the course of experimental immune-mediated demyelinating diseases. Immunol Rev vol. 144, p. 225-44, 1995.

Miner JH. A molecular look at the glomerular barrier. Nephron Exp Nephrol vol. 94, p. e119-22, 2003.

Moeller MJ, Soofi A, Braun GS, Li X, Watzl C, Kriz W, Holzman LB. Protocadherin FAT1 binds Ena/VASP proteins and is necessary for actin dynamics and cell polarization. EMBO J vol. 23, p. 3769-79, 2004.

Mogensen CE, Christensen CK. Predicting diabetic nephropathy in insulin-dependent patients. N Engl J Med vol. 311, p. 89-93, 1984.

Murata E, Tashiro S, Hiraoka T. Streptozotocin effective for treating multiple-hormone-producing malignant islet cell tumor. Jpn J Surg vol. 15, p. 63-7, 1985.

Mykkanen OM, Gronholm M, Ronty M, Lalowski M, Salmikangas P, Suila H, Carpen O. Characterization of human palladin, a microfilament-associated protein. Mol Biol Cell vol. 12, p. 3060-73, 2001.

Nakhooda AF, Like AA, Chappel CI, Murray FT, Marliss EB. The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. Diabetes vol. 26, p. 100-12, 1977.

Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC, Elliston K, Stern D, Shaw A. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. J Biol Chem vol. 267, p. 14998-5004, 1992.

Netter FH. Atlas of Human Anatomy. 9th ed. Novartis, p. 315-33, 1997.

Nicoloff G, Blazhev A, Petrova C, Christova P. Circulating immune complexes among diabetic children. Clin Dev Immunol vol. 11, p. 61-6, 2004.

Nishi S, Ueno M, Hisaki S, Iino N, Iguchi S, Oyama Y, Imai N, Arakawa M, Gejyo F. Ultrastructural characteristics of diabetic nephropathy. Med Electron Microsc vol. 33, p. 65-73, 2000.

Nistico L, Buzzetti R, Pritchard LE, Van der Auwera B, Giovannini C, Bosi E, Larrad MT, Rios MS, Chow CC, Cockram CS, Jacobs K, Mijovic C, Bain SC, Barnett AH, Vandewalle CL, Schuit F, Gorus FK, Tosi R, Pozzilli P, Todd JA. The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry. Hum Mol Genet vol. 5, p. 1075-80, 1996.

Nordwall M, Bojestig M, Arnqvist HJ, Ludvigsson J, Linkoping Diabetes Complications Study. Declining incidence of severe retinopathy and persisting decrease of nephropathy in an unselected population of Type 1 diabetes-the Linkoping Diabetes Complications Study. Diabetologia vol. 47, p. 1266-72, 2004.

Ohashi PS, Oehen S, Buerki K, Pircher H, Ohashi CT, Odermatt B, Malissen B, Zinkernagel RM, Hengartner H. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell vol. 65, p. 305-17, 1991.

Okada S, Shikata K, Matsuda M, Ogawa D, Usui H, Kido Y, Nagase R, Wada J, Shikata Y, Makino H. Intercellular adhesion molecule-1-deficient mice are resistant against renal injury after induction of diabetes. Diabetes vol. 52, p. 2586-93, 2003.

Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell vol. 65, p. 319-31, 1991.

Onengut-Gumuscu S, Ewens KG, Spielman RS, Concannon P. A functional polymorphism (1858C/T) in the PTPN22 gene is linked and associated with type I diabetes in multiplex families. Genes Immun vol. 5, p. 678-80, 2004.

Onkamo P, Vaananen S, Karvonen M, Tuomilehto J. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. Diabetologia vol. 42, p. 1395-403, 1999.

Orikasa M, Matsui K, Oite T, Shimizu F. Massive proteinuria induced in rats by a single intravenous injection of a monoclonal antibody. J Immunol vol. 141, p. 807-14, 1988.

Otey CA, Vasquez GB, Burridge K, Erickson BW. Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain. J Biol Chem vol. 268, p. 21193-7, 1993.

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

Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL. Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science vol. 222, p. 1337-9, 1983.

Park CW, Kim JH, Lee JH, Kim YS, Ahn HJ, Shin YS, Kim SY, Choi EJ, Chang YS, Bang BK. High glucose-induced intercellular adhesion molecule-1 (ICAM-1) expression through an osmotic effect in rat mesangial cells is PKC-NF-kappa B-dependent. Diabetologia vol. 43, p. 1544-53, 2000.

Parving HH, Oxenboll B, Svendsen PA, Christiansen JS, Andersen AR. Early detection of patients at risk of developing diabetic nephropathy. A longitudinal study of urinary albumin excretion. Acta Endocrinol (Copenh) vol. 100, p. 550-5, 1982.

Patari A, Forsblom C, Havana M, Taipale H, Groop PH, Holthofer H. Nephrinuria in Diabetic Nephropathy of Type 1 Diabetes. Diabetes vol. 52, p. 2969-74, 2003.

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

Patrie KM, Drescher AJ, Goyal M, Wiggins RC, Margolis B. The membrane-associated guanylate kinase protein MAGI-1 binds megalin and is present in glomerular podocytes. J Am Soc Nephrol vol. 12, p. 667-77, 2001.

Petermann AT, Pippin J, Krofft R, Blonski M, Griffin S, Durvasula R, Shankland SJ. Viable podocytes detach in experimental diabetic nephropathy: potential mechanism underlying glomerulosclerosis. Nephron Exp Nephrol vol. 98, p. e114-23, 2004.

Pettitt DJ, Saad MF, Bennett PH, Nelson RG, Knowler WC. Familial predisposition to renal disease in two generations of Pima Indians with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia vol. 33, p. 438-43, 1990.

Pugliese A, Zeller M, Fernandez A,Jr, Zalcberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST, Patel DD. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat Genet vol. 15, p. 293-7, 1997.

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

Pyke C, Kristensen P, Ostergaard PB, Oturai PS, Romer J. Proteoglycan expression in the normal rat kidney. Nephron vol. 77, p. 461-70, 1997.

Raats CJ, van den Born J, Bakker MA, Oppers-Walgreen B, Pisa BJ, Dijkman HB, Assmann KJ, Berden JH. Expression of agrin, dystroglycan, and utrophin in normal renal tissue and in experimental glomerulopathies. Am J Pathol vol. 156, p. 1749-65, 2000.

Rakieten N, Gordon BS, Cooney DA, Davis RD, Schein PS. Renal tumorigenic action of streptozotocin (NSC-85998) in rats. Cancer Chemother Rep vol. 52, p. 563-7, 1968.

Rakieten N, Rakieten ML, Nadkarni MV. Studies on the diabetogenic action of streptozotocin (NSC-37917). Cancer Chemother Rep vol. 29, p. 91-8, 1963.

Ramana KV, Friedrich B, Tammali R, West MB, Bhatnagar A, Srivastava SK. Requirement of aldose reductase for the hyperglycemic activation of protein kinase C and formation of diacylglycerol in vascular smooth muscle cells. Diabetes vol. 54, p. 818-29, 2005.

Regele HM, Fillipovic E, Langer B, Poczewki H, Kraxberger I, Bittner RE, Kerjaschki D. Glomerular expression of dystroglycans is reduced in minimal change nephrosis but not in focal segmental glomerulosclerosis. J Am Soc Nephrol vol. 11, p. 403-12, 2000.

Regoli M, Bendayan M. Alterations in the expression of the alpha 3 beta 1 integrin in certain membrane domains of the glomerular epithelial cells (podocytes) in diabetes mellitus. Diabetologia vol. 40, p. 15-22, 1997.

Reiser J, Polu KR, Moller CC, Kenlan P, Altintas MM, Wei C, Faul C, Herbert S, Villegas I, Avila-Casado C, McGee M, Sugimoto H, Brown D, Kalluri R, Mundel P, Smith PL, Clapham DE, Pollak MR. TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. Nat Genet vol. 37, p. 739-44, 2005.

Reiser J, Kriz W, Kretzler M, Mundel P. The glomerular slit diaphragm is a modified adherens junction. J Am Soc Nephrol vol. 11, p. 1-8, 2000.

Reivinen J, Holthofer H, Miettinen A. O-acetyl GD3 ganglioside in human peripheral blood T lymphocytes. Int Immunol vol. 6, p. 1409-16, 1994.

Ridgway WM. The non obese diabetic (NOD) mouse: a unique model for understanding the interaction between genetics and T cell responses. Rev Endocr Metab Disord vol. 4, p. 263-9, 2003.

Ries F, Klastersky J. Nephrotoxicity induced by cancer chemotherapy with special emphasis on cisplatin toxicity. Am J Kidney Dis vol. 8, p. 368-79, 1986.

Roep BO. The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure. Diabetologia vol. 46, p. 305-21, 2003.

Roll U, Nuber A, Schroder A, Gerlach E, Janka HU, Ziegler AG. No association of antibodies to glutamic acid decarboxylase and diabetic complications in patients with IDDM. Diabetes Care vol. 18, p. 210-5, 1995.

Roselli S, Gribouval O, Boute N, Sich M, Benessy F, Attie T, Gubler MC, Antignac C. Podocin localizes in the kidney to the slit diaphragm area. Am J Pathol vol. 160, p. 131-9, 2002.

Rosen ED, Kulkarni RN, Sarraf P, Ozcan U, Okada T, Hsu CH, Eisenman D, Magnuson MA, Gonzalez FJ, Kahn CR, Spiegelman BM. Targeted elimination of peroxisome proliferator-activated receptor gamma in beta cells leads to abnormalities in islet mass without compromising glucose homeostasis. Mol Cell Biol vol. 23, p. 7222-9, 2003.

Rossing P, Hougaard P, Parving HH. Risk factors for development of incipient and overt diabetic nephropathy in type 1 diabetic patients: a 10-year prospective observational study. Diabetes Care vol. 25, p. 859-64, 2002.

Rossing P, Rossing K, Jacobsen P, Parving HH. Unchanged incidence of diabetic nephropathy in IDDM patients. Diabetes vol. 44, p. 739-43, 1995.

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

Ryan GB, Karnovsky MJ. An ultrastructural study of the mechanisms of proteinuria in aminonucleoside nephrosis. Kidney Int vol. 8, p. 219-32, 1975.

Sadoff L. Nephrotoxicity of streptozotocin (NSC-85998). Cancer Chemother Rep vol. 54, p. 457-9, 1970.

Sale MM, Freedman BI. Genetic determinants of albuminuria and renal disease in diabetes mellitus. Nephrol Dial Transplant vol. 21, p. 13-6, 2006.

Saraheimo M, Teppo AM, Forsblom C, Fagerudd J, Groop PH. Diabetic nephropathy is associated with low-grade inflammation in Type 1 diabetic patients. Diabetologia vol. 46, p. 1402-7, 2003.

Savola K, Bonifacio E, Sabbah E, Kulmala P, Vahasalo P, Karjalainen J, Tuomilehto-Wolf E, Merilainen J, Akerblom HK, Knip M. IA-2 antibodies--a sensitive marker of IDDM with clinical onset in childhood and adolescence. Childhood Diabetes in Finland Study Group. Diabetologia vol. 41, p. 424-9, 1998.

Schnabel E, Anderson JM, Farquhar MG. The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerular epithelium. J Cell Biol vol. 111, p. 1255-63, 1990.

Schnedl WJ, Ferber S, Johnson JH, Newgard CB. STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes vol. 43, p. 1326-33, 1994.

Schrijvers BF, Flyvbjerg A, De Vriese AS. The role of vascular endothelial growth factor (VEGF) in renal pathophysiology. Kidney Int vol. 65, p. 2003-17, 2004.

Schwartz BD. The Human Major Histocompatibility HLA Complex. In: Stites DP, Stobo JD, Wells JV, editors. Basic & Clinical Immunology. 6th ed. Appleton & Lange, p. 50-64, 1987.

Schwarz K, Simons M, Reiser J, Saleem MA, Faul C, Kriz W, Shaw AS, Holzman LB, Mundel P. Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. J Clin Invest vol. 108, p. 1621-9, 2001.

Seaquist ER, Goetz FC, Rich S, Barbosa J. Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy. N Engl J Med vol. 320, p. 1161-5, 1989.

Sellin L, Huber TB, Gerke P, Quack I, Pavenstadt H, Walz G. NEPH1 defines a novel family of podocin interacting proteins. FASEB J vol. 17, p. 115-7, 2003.

Shih NY, Li J, Cotran R, Mundel P, Miner JH, Shaw AS. CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. Am J Pathol vol. 159, p. 2303-8, 2001.

Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, Miner JH, Shaw AS. Congenital nephrotic syndrome in mice lacking CD2-associated protein. Science vol. 286, p. 312-5, 1999.

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

Smedsrod B, Melkko J, Araki N, Sano H, Horiuchi S. Advanced glycation end products are eliminated by scavenger-receptor-mediated endocytosis in hepatic sinusoidal Kupffer and endothelial cells. Biochem J vol. 322 (Pt 2), p. 567-73, 1997.

Sohnlein P, Muller M, Syren K, Hartmann U, Bohm BO, Meinck HM, Knip M, Akerblom HK, Richter W. Epitope spreading and a varying but not disease-specific GAD65 antibody response in Type I diabetes. The Childhood Diabetes in Finland Study Group. Diabetologia vol. 43, p. 210-7, 2000.

Steffes MW, Schmidt D, McCrery R, Basgen JM, International Diabetic Nephropathy Study Group. Glomerular cell number in normal subjects and in type

1 diabetic patients. Kidney Int vol. 59, p. 2104-13, 2001.

Stockand JD, Sansom SC. Glomerular mesangial cells: electrophysiology and regulation of contraction. Physiol Rev vol. 78, p. 723-44, 1998.

Sun Y, Patrakka J, Bjorklund M, Koivunen E, Tryggvason K. In: Screening of a phage library with human nephrin reveals MEGF1/Fat2 as a novel component of the glomerular podocyte slit diaphragm. American Society of Nephrology Abstracts: SA-FC119, 2005.

Suzuki T, Takemura H, Noiri E, Nosaka K, Toda A, Taniguchi S, Uchida K, Fujita T, Kimura S, Nakao A. Puromycin aminonucleoside induces apoptosis and increases HNE in cultured glomerular epithelial cells. Free Radic Biol Med vol. 31, p. 615-23, 2001.

Svensson M, Sundkvist G, Arnqvist HJ, Bjork E, Blohme G, Bolinder J, Henricsson M, Nystrom L, Torffvit O, Waernbaum I, Ostman J, Eriksson JW, Diabetes Incidence Study in Sweden (DISS). Signs of nephropathy may occur early in young adults with diabetes despite modern diabetes management: results from the nationwide population-based Diabetes Incidence Study in Sweden (DISS). Diabetes Care vol. 26, p. 2903-9, 2003.

Takeda T. Podocyte cytoskeleton is connected to the integral membrane protein podocalyxin through Na+/H+-exchanger regulatory factor 2 and ezrin. Clin Exp Nephrol vol. 7, p. 260-9, 2003.

Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature vol. 372, p. 182-6, 1994.

Tanaka S, Avigad G, Brodsky B, Eikenberry EF. Glycation induces expansion of the molecular packing of collagen. J Mol Biol vol. 203, p. 495-505, 1988.

Thomas PE, Wharram BL, Goyal M, Wiggins JE, Holzman LB, Wiggins RC. GLEPP1, a renal glomerular epithelial cell (podocyte) membrane protein-tyrosine phosphatase. Identification, molecular cloning, and characterization in rabbit. J Biol Chem vol. 269, p. 19953-62, 1994.

Thorn LM, Forsblom C, Fagerudd J, Thomas MC, Pettersson-Fernholm K, Saraheimo M, Waden J, Ronnback M, Rosengard-Barlund M, Bjorkesten CG, Taskinen MR, Groop PH, FinnDiane Study Group. Metabolic syndrome in type 1 diabetes: association with diabetic nephropathy and glycemic control (the FinnDiane study). Diabetes Care vol. 28, p. 2019-24, 2005.

Thornalley PJ. The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem J vol. 269, p. 1-11, 1990.

Tischer CC, Madsen KM. Anatomy of the Kidney. In: Brenner BM, Rector FC, editors. The Kidney. 4th ed. W.B. Saunders Company, 1991.

Tomura S, Kawada K, Saito K, Lin YL, Endou K, Hirano C, Yanagi H, Tsuchiya S, Shiba K. Prevalence of microalbuminuria and relationship to the risk of cardiovascular disease in the Japanese population. Am J Nephrol vol. 19, p. 13-20, 1999.

Topham PS, Kawachi H, Haydar SA, Chugh S, Addona TA, Charron KB, Holzman LB, Shia M, Shimizu F, Salant DJ. Nephritogenic mAb 5-1-6 is directed at the extracellular domain of rat nephrin. J Clin Invest vol. 104, p. 1559-66, 1999.

Toyoda M, Suzuki D, Umezono T, Uehara G, Maruyama M, Honma M, Sakai T, Sakai H. Expression of human nephrin mRNA in diabetic nephropathy. Nephrol Dial Transplant vol. 19, p. 380-5, 2004.

Trouet D, Lennon R, Ni L, Mathieson PW, Saleem MA. In: Downregulation of TRPC6 by intact nephrin in the presence of human plasma- A protective mechanism for maintenance of podocyte process structure? American Society of Nephrology Abstracts: F-PO456, 2005.

Tryggvason G, Indridason OS, Thorsson AV, Hreidarsson AB, Palsson R. Unchanged incidence of diabetic nephropathy in Type 1 diabetes: a nation-wide study in Iceland. Diabet Med vol. 22, p. 182-7, 2005.

Tsilibary EC, Charonis AS, Reger LA, Wohlhueter RM, Furcht LT. The effect of nonenzymatic glucosylation on the binding of the main noncollagenous NC1 domain to type IV collagen. J Biol Chem vol. 263, p. 4302-8, 1988.

Tuohy VK, Yu M, Yin L, Kawczak JA, Kinkel RP. Spontaneous regression of primary autoreactivity during chronic progression of experimental autoimmune encephalomyelitis and multiple sclerosis. J Exp Med vol. 189, p. 1033-42, 1999.

Tuttle KR. Linking metabolism and immunology: diabetic nephropathy is an inflammatory disease. J Am Soc Nephrol vol. 16, p. 1537-8, 2005.

Undlien DE, Kockum I, Ronningen KS, Lowe R, Saanjeevi CB, Graham J, Lie BA, Akselsen HE, Lernmark A, Thorsby E. HLA associations in type 1 diabetes among patients not carrying high-risk DR3-DQ2 or DR4-DQ8 haplotypes. Tissue Antigens vol. 54, p. 543-51, 1999.

United Kingdom Renal Registry. UK Renal Registry Report 2003. Annual Data Report. U.K. Renal Registry United Kingdom, 2003.

United States Renal Data System. USRDS 2003 Annual Data Report: Atlas of End-Stage Renal Disease in the United States. Annual Data Report. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases USA, 2003.

Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E, Polychronakos C. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. Nat Genet vol. 15, p. 289-92, 1997.

Vavra JJ, Deboer C, Dietz A, Hanka LJ, Sokolski WT. Streptozotocin, a new antibacterial antibiotic. Antibiot Annu vol. 7, p. 230-5, 1959.

Vernier RL, Papermaster BW, Good RA. Aminonucleoside nephrosis. I. Electron microscopic study of the renal lesion in rats. J Exp Med vol. 109, p. 115-26, 1959.

Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet vol. 1, p. 1430-2, 1982.

Vinik AI, Leichter SB, Pittenger GL, Stansberry KB, Holland MT, Powers AC, Suwanwalaikorn S. Phospholipid and glutamic acid decarboxylase autoantibodies in diabetic neuropathy. Diabetes Care vol. 18, p. 1225-32, 1995.

Vlassara H, Li YM, Imani F, Wojciechowicz D, Yang Z, Liu FT, Cerami A. Identification of galectin-3 as a high-affinity binding protein for advanced glycation end products (AGE): a new member of the AGE-receptor complex. Mol Med vol. 1, p. 634-46, 1995.

Vyse TJ, Todd JA. Genetic analysis of autoimmune disease. Cell vol. 85, p. 311-8, 1996.

Walikonis RS, Oguni A, Khorosheva EM, Jeng CJ, Asuncion FJ, Kennedy MB. Densin-180 forms a ternary complex with the (alpha)-subunit of Ca2+/calmodulin-dependent protein kinase II and (alpha)-actinin. J Neurosci vol. 21, p. 423-33, 2001.

Wang SX, Ahola H, Palmen T, Solin ML, Luimula P, Holthofer H. Recurrence of nephrotic syndrome after transplantation in CNF is due to autoantibodies to nephrin. Exp Nephrol vol. 9, p. 327-31, 2001a.

Wang SX, Mene P, Holthofer H. Nephrin mRNA regulation by protein kinase C. J Nephrol vol. 14, p. 98-103, 2001b.

Wang W, Koka V, Lan HY. Transforming growth factor-beta and Smad signalling in kidney diseases. Nephrology (Carlton) vol. 10, p. 48-56, 2005.

Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW. Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. Biochemistry vol. 34, p. 3702-9, 1995.

Wendt TM, Tanji N, Guo J, Kislinger TR, Qu W, Lu Y, Bucciarelli LG, Rong LL, Moser B, Markowitz GS, Stein G, Bierhaus A, Liliensiek B, Arnold B, Nawroth PP, Stern DM, D'Agati VD, Schmidt AM. RAGE drives the development of glomerulosclerosis and implicates podocyte activation in the pathogenesis of diabetic nephropathy. Am J Pathol vol. 162, p. 1123-37, 2003.

Wentworth BM, Schaefer IM, Villa-Komaroff L, Chirgwin JM. Characterization of the two nonallelic genes encoding mouse preproinsulin. J Mol Evol vol. 23, p. 305-12, 1986.

Wharram BL, Goyal M, Gillespie PJ, Wiggins JE, Kershaw DB, Holzman LB, Dysko RC, Saunders TL, Samuelson LC, Wiggins RC. Altered podocyte structure in GLEPP1 (Ptpro)-deficient mice associated with hypertension and low glomerular filtration rate. J Clin Invest vol. 106, p. 1281-90, 2000.

Willems D, Dorchy H, Dufrasne D. Serum antioxidant status and oxidized LDL in well-controlled young type 1 diabetic patients with and without subclinical complications. Atherosclerosis vol. 137 Suppl, p. S61-4, 1998.

Williams G, Monson JP. Diabetes Mellitus and Disorders of Lipid and Intermediary Metabolism. In: Souhami RL, Moxham J, editors. Textbook of Medicine. 2nd ed. Churchill Livingstone, p. 744-6, 1994.

Winn MP, Conlon PJ, Lynn KL, Farrington MK, Creazzo T, Hawkins AF, Daskalakis N, Kwan SY, Ebersviller S, Burchette JL, Pericak-Vance MA, Howell DN, Vance JM, Rosenberg PB. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. Science vol. 308, p. 1801-4, 2005.

Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF. Disruption of IRS-2 causes type 2 diabetes in mice. Nature vol. 391, p. 900-4, 1998.

Wolf G, Ziyadeh FN. The role of angiotensin II in diabetic nephropathy: emphasis on nonhemodynamic mechanisms. Am J Kidney Dis vol. 29, p. 153-63, 1997.

Xu ZG, Ryu DR, Yoo TH, Jung DS, Kim JJ, Kim HJ, Choi HY, Kim JS, Adler SG, Natarajan R, Han DS, Kang SW. P-Cadherin is decreased in diabetic glomeruli and in glucose-stimulated podocytes in vivo and in vitro studies. Nephrol Dial Transplant vol. 20, p. 524-31, 2005.

Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, Pinsky D, Stern D. Enhanced cellular oxidant stress by the interaction of advanced glycation end

products with their receptors/binding proteins. J Biol Chem vol. 269, p. 9889-97, 1994.

Yaoita E, Sato N, Yoshida Y, Nameta M, Yamamoto T. Cadherin and catenin staining in podocytes in development and puromycin aminonucleoside nephrosis. Nephrol Dial Transplant vol. 17 Suppl 9, p. 16-9, 2002.

Yoshiji H, Kuriyama S, Fukui H. Perindopril: possible use in cancer therapy. Anticancer Drugs vol. 13, p. 221-8, 2002.

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

Zheng W, She JX. Genetic association between a lymphoid tyrosine phosphatase (PTPN22) and type 1 diabetes. Diabetes vol. 54, p. 906-8, 2005.

Zhou G, Li C, Cai L. Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor beta-independent pathway. Am J Pathol vol. 165, p. 2033-43, 2004.

Ziyadeh FN. Mediators of diabetic renal disease: the case for tgf-Beta as the major mediator. J Am Soc Nephrol vol. 15 Suppl 1, p. S55-7, 2004.

9 ORIGINAL PUBLICATIONS