Division of Nephrology
Helsinki University Central Hospital
Helsinki, Finland

and

Folkhälsan Research Center
Folkhälsan Institute of Genetics
University of Helsinki
Helsinki, Finland

SUSCEPTIBILITY GENES FOR DIABETIC NEPHROPATHY IN TYPE 1 DIABETES

Studies of five genes involved in glucose metabolism,
glomerular structure, and blood pressure regulation

Kim Pettersson-Fernholm

ACADEMIC DISSERTATION

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Supervisor   Per-Henrik Groop, DMSc
                              Docent
                              Folkhälsan Research Center
                              University of Helsinki
                              Helsinki, Finland

Reviewers   Markku Laakso, DMSc
                              Professor
                              Department of Medicine
                              University of Kuopio
                              Kuopio, Finland

                              and

                              Aarno Palotie, MD, PhD
                              Professor
                              The Finnish Genome Center
                              University of Helsinki
                              Helsinki, Finland

Opponent   Alexander P. Maxwell, PhD, FRCPC, FRCP
                              Professor
                              Queen's University of Belfast
                              Belfast, Northern Ireland

CONTENTS

Everything is possible unless proven to be impossible – yet it may be impossible only for the moment.

Pearl S. Buck

To Johanna and Sofia

ORIGINAL PUBLICATIONS

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

I   Pettersson-Fernholm K, Forsblom C, Hudson B, Grant PJ, Perola M, Groop PH, for the FinnDiane Study Group: The functional –374 T/A RAGE gene polymorphism is associated with proteinuria and cardiovascular disease in type 1 diabetic patients. Diabetes 52(3):891-4, 2003.

II   Pettersson-Fernholm K, Forsblom C, Perola M, Groop PH, for the FinnDiane Study Group: Polymorphisms in the nephrin gene and diabetic nephropathy in type 1 diabetic patients. Kidney Int 63(4):1205-10, 2003.

III   Pettersson-Fernholm K, Karvonen MK, Kallio J, Forsblom C, Koulu M, Pesonen U, Fagerudd J, Groop PH, for the FinnDiane Study Group: Leucine 7 to Proline 7 polymorphism in the Prepromeuropeptide Y gene is associated with proteinuria, coronary heart disease and glycemic control in type 1 diabetic patients. Diabetes Care 27(2):503-9, 2004.

IV   Pettersson-Fernholm KJ, Forsblom CM, Perola M, Fagerudd JA, Groop PH, for the FinnDiane Study Group: Dopamine D3 receptor gene polymorphisms, blood pressure and nephropathy in type 1 diabetic patients. Nephrol Dial Transplant 19(6):1432-6, 2004.

V   Pettersson-Fernholm K, Fröjdö S, Fagerudd J, Thomas MC, Forsblom CM, Groop PH, for the FinnDiane Study Group: The X-chromosomal angiotensin II type 2 receptor gene is associated with a gender-specific effect on kidney function and pulse pressure in patients with type 1 diabetes (submitted).

ABBREVIATIONS

ACE	angiotensin-converting enzyme
AGEs	advanced glycation end-products
AHT	antihypertensive treatment
AMI	acute myocardial infarction
AT1	angiotensin II type 1 receptor
AT2	angiotensin II type 2 receptor
BP	blood pressure
BMI	body mass index
CHD	coronary heart disease
CVD	cardiovascular disease
DBP	diastolic blood pressure
DNA	deoxyribonucleic acid
DRD3	dopamine receptor 3
eGDR	estimated glucose disposal rate
ESRD	end-stage renal disease
GBM	glomerular basement membrane
GFR	glomerular filtration rate estimated according to Cockcroft-Gault formula
GH	growth hormone
HbA_{1c}	glycosylated hemoglobin A_{1c}
HDL	high-density lipoprotein
IGF	insulin-like growth factor
IL	interleukin
LD	linkage disequilibrium
LDL	low-density lipoprotein
MICRO	microalbuminuria
NORMO	normal urinary albumin excretion rate
NPY	neuropeptide Y
NS	not statistically significant ($p \geq 0.05$)
HSPG	hepararan sulphate proteoglycan (Perlecan)
PKC	protein kinase C
PCR	polymerase chain reaction
PP	pulse pressure
PROT	proteinuria
PVD	peripheral vascular disease
RAAS	renin-angiotensin-aldosterone system
RAGE	receptor for advanced glycation end-products
SBP	systolic blood pressure
SD	standard deviation
SEM	standard error of mean
TGF	transforming growth factor
UAER	urinary albumin excretion rate
VEGF	vascular endothelial growth factor
WHR	waist-to-hip ratio

ABSTRACT

Introduction: A third of all patients with type 1 diabetes develop diabetic nephropathy within 10 to 30 years, which leads to a decline in renal function and eventually to dialysis or renal transplantation. This complication is associated with a 40-fold increase in the incidence of cardiovascular disease and premature death. Substantial evidence exists for genetic factors in the development and progression of the disease. The present study was undertaken to investigate gene variations that may play a role in the pathogenesis of diabetic nephropathy and its related traits.

Subjects and methods: The study uses a cross-sectional, case-control design comprising 996 patients with type 1 diabetes. All patients were recruited from 20 referral centers as a part of a nationwide multi-center study, the FinnDiane study. They were classified into four groups according to their urinary albumin excretion rate. A number of previously characterized polymorphisms in the genes investigated were selected for each study. The polymorphic region was amplified by polymerase chain reaction, and genotyping was performed by a solid-phase minisequencing method.

Results: The AA genotype of the functional –374 T/A polymorphism in the *RAGE* gene located on chromosome 6p21.3 had a protective effect against proteinuria in patients with poor glycemic control, and against macrovascular disease. The Pro7 allele of the Leu7Pro polymorphism of preproneuropeptide Y on chromosome 7p15.1 was associated with poor glycemic control, proteinuria, higher triglycerides, and coronary heart disease. The AA haplotype of the X-chromosomal *AT2* gene was associated with a decline in renal function and an increase in pulse pressure in males. Polymorphisms of the nephrin and the *DRD3* gene on chromosome 3q13.3 associated neither with diabetic nephropathy, amount of proteinuria, serum lipids nor blood pressure.

Conclusion: This study suggests a gene–environment effect between the *RAGE* gene and diabetic nephropathy. The *AT2* gene may play a role in the male predominance of diabetic nephropathy by influencing renal function and premature aging of the vascular tree. The Pro7 allele of preproneuropeptide Y may be involved in the pathogenesis of diabetic nephropathy through an impairment of glycemic control and lipids. No evidence appeared for any involvement of the *DRD3* or the nephrin gene in diabetic late complications.

1. INTRODUCTION

Diabetes mellitus was first described by Aretaeus of Cappadocia 2000 years ago (1). Diabetes is a Greek word meaning "going through" used together with the Latin *mellitus* originating from the Greek *meli*, meaning "sweet" or "honey." The words thus describe remarkably well the main symptoms of the disease, i.e., how a major leakage of glucose into the urine causes massive urine volumes, resulting in excessive body fluid deprivation and thirst. This much-feared disease, which is caused by global destruction of the insulin-producing islet cells in the pancreas, was untreatable and relentless, resulting in ketoacidosis, cachexia, and death within 1 to 2 years, until the discovery of insulin by Banting and Best in 1922. The novel treatment with exogenous insulin was doubtless one of the major achievements in medicine during the 20th century.

However, although exogenous insulin saved the patients from early death, the treatment could not entirely mimic the physiological insulin production. Consequently, the body was still exposed to a more or less constant mild or moderate hyperglycemia. Soon it was observed that decades of supraphysiological blood glucose levels in a subset of patients had detrimental effects on the peripheral nerves, heart, eyes, blood vessels, and kidneys. An entirely new kidney disease had entered the picture and was clinically characterized by elevated blood pressure (BP), proteinuria, and edema, eventually leading to renal failure and uremia. Specific histological changes in the kidney were soon discovered, and diabetic glomerulosclerosis (diabetic nephropathy) was first described by Kimmelstiel and Wilson in 1936 (2). Patients with diabetic nephropathy also showed a dramatically increased mortality due to cardiovascular disease, more diabetic eye disease (retinopathy) causing blindness, and more peripheral nerve destruction (neuropathy) resulting in foot ulcers and amputation.

Interestingly, only one-third of the patients with type 1 diabetes will eventually develop clinically manifest nephropathy (3,4). Although the presence of hyperglycemia is crucial for disease development, it cannot alone or even together with a number of other environmental factors explain this pattern. Clinicians treating type 1 diabetic patients have always been perplexed by the fact that some patients do not develop any late diabetic complications despite decades of even very poor glycemic control, whereas others may develop progressive diabetic nephropathy despite fairly good glycemic control. Today, evidence is convincing for genetic predisposition to the disease, as shown by large family studies (5) and as indicated by a characteristic incidence peak of

nephropathy after 15 to 20 years of diabetes (6). These studies, together with several others, are clearly pointing towards the presence of susceptibility genes in the pathogenesis of diabetic nephropathy.

This study was undertaken to explore in patients with type 1 diabetes biologically plausible candidate genes coding for proteins that could interfere with mechanisms leading to the initiation or the progression of diabetic nephropathy and other late complications. This study utilizes a large, unique national cohort of almost 1000 patients that is part of the FinnDiane study.

2. REVIEW OF THE LITERATURE

2.1. Classification of diabetes and its late complications

Diabetes mellitus, a severe health problem with an incidence rapidly increasing worldwide, comprises two distinct major types. Approximately 10% of all diabetic patients suffer from type 1 diabetes, formerly called insulin-dependent diabetes mellitus (IDDM) or juvenile onset diabetes. It is characterized by a progressive autoimmune destruction of the insulin-producing islets in the pancreas, resulting in a complete loss of insulin secretion. This is in total contrast with the considerably more common type 2 diabetes, previously known as non-insulin dependent diabetes mellitus (NIDDM) or as maturity onset diabetes mellitus. This disease is in the majority of cases characterized by the metabolic syndrome, which includes central obesity, hypertension, dyslipidemia, impaired fibrinolysis, hyperuricemia, and insulin resistance, and furthermore, by increased gluconeogenesis and eventually insufficient insulin production. In addition to these two major subtypes there is a plethora of rarer forms of diabetes, which together account for less than some 5 to 10% of all diabetes subtypes (7,8).

Patients with both type 1 and type 2 diabetes show a substantially increased risk for late complications. A common denominator for all long-term microvascular and macrovascular complications is extensive vascular damage. In macrovascular complications, accelerated atherosclerosis results in cardiovascular disease (CVD) such as coronary heart disease (CHD) and acute myocardial infarction (AMI). In the lower extremities, macrovascular disease manifests as peripheral vascular disease (PVD) resulting in gangrene and amputation, whereas the consequence of thrombosis in a brain vessel is stroke. Microvascular complications comprise changes in the small blood vessels of the eye that result in diabetic retinopathy, in the peripheral nerves, causing neuropathy, and finally in the kidney, causing diabetic glomerulosclerosis or diabetic nephropathy.

2.2. Diabetic nephropathy in type 1 diabetes

2.2.1. Epidemiology and natural history

Only 30 to 45% of patients with type 1 diabetes will develop diabetic nephropathy (3,4,6), although lower figures also occur (9). Incidence of diabetic nephropathy is at its peak after 10 to 15 years following the onset of diabetes (2-3%) but falls to 0.5 to 1% after 20 years

of diabetes (10,11).

Figure 1 shows the natural history of diabetic nephropathy. Its standard classification includes three stages according to urinary albumin excretion rate (UAER): 1) incipient nephropathy (microalbuminuria, UAER in an overnight urinary collection 20-200 µg/min) lasting 5 to 15 years, 2) overt nephropathy (proteinuria or macroalbuminuria, UAER > 200 µg/min) lasting 5 to 10 years, and 3) end-stage renal disease (ESRD) lasting 3 to 6 years without dialysis or renal transplantation. Mogensen a few years ago suggested a five-stage classification by adding two stages that precede microalbuminuria (12). The first includes enlargement of the kidneys (nephromegaly) as well as an increase in glomerular filtration rate (hyperfiltration), and the second a transient period of normal UAER. This latter stage can be normalized by insulin treatment (13). Since hyperfiltration predicts the development of diabetic nephropathy, it is considered necessary, but not sufficient, for progression of the disease (14). Although the first clinically applicable sign of diabetic nephropathy is microalbuminuria, evidence exists that UAER already in the upper normal range (10-20 µg/min) can predict progression to overt nephropathy (15,16).
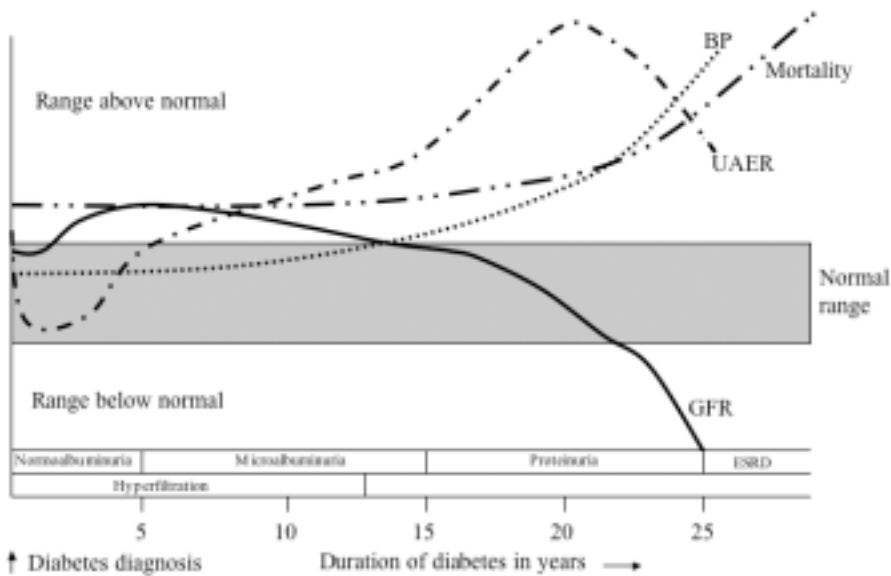
Figure 1. Natural history of diabetic nephropathy in type 1 diabetes. Adapted from Jerums *et al.* (451). For abbreviations, see page 7.

Microalbuminuria is a strong predictor for overt diabetic nephropathy: Several studies have shown that 75 to 100% of microalbuminuric patients develop diabetic nephropathy within 6 to 14 years (17-19). Although after 20 years of diabetes duration its predictive value decreases (11), risk is 20-fold higher than in patients with normal UAER. According to one recent study by Perkins *et al*. (20), however, the predictive value of microalbuminuria is significantly lower, since they observed a 6-year cumulative regression incidence of 58% from microalbuminuria to normal UAER. When microalbuminuria is slowly progressing to proteinuria, a significant increase in BP occurs (21,22), although renal function at this stage is usually normal. As the amount of proteinuria is increasing, concurrently glomerular filtration rate is declining at an average 10 ml/min/year (22,23). Within 5 to 10 years, the patients enter ESRD, characterized by total loss of renal function and clinically manifest uremia.

2.2.2. Comorbidity and mortality

Microalbuminuria also predicts proliferative retinopathy (24), whereas overt nephropathy predicts retinal disease resulting in loss of vision (25). Patients with diabetic nephropathy thus usually have diabetic retinopathy. However, although nearly all type 1 diabetic patients eventually develop retinopathy, only a third will develop overt diabetic nephropathy (26). Consequently, UAER increases exponentially with elevated BP, whereas UAER does not increase with elevated BP in patients without retinopathy.

If diabetic patients are treated with insulin alone, their mortality rises to 50% within 7 years after development of proteinuria (3). The prognosis for a patient with type 1 diabetes is largely dependent on the development of diabetic nephropathy. There is already a 2-fold increase in relative mortality for patients without nephropathy, but a 40-fold increase for those with proteinuria and at maximum a 100-fold risk at age 35 due to cardiovascular disease (27). These figures are supported by more recent figures for Finnish type 1 diabetic patients, showing a 10-fold increased risk for patients with nephropathy in comparison to those without this complication (28).

2.2.3. Effect of treatment on progression and prognosis

During the last 20 years the intensified treatment of patients with ESRD, proteinuria, and microalbuminuria has led to a substantial improvement in their long-term prognosis.

Diabetic nephropathy does not occur in the absence of hyperglycemia. The

Diabetes Control and Complications Trial (DCCT) showed that risk for diabetic nephropathy depends upon blood glucose concentrations, *i.e.*, the risk per unit increase in glycosylated hemoglobin A_{1c} (HbA$_{1c}$), is greater at high levels than at low levels (29). Such a curvilinear relationship between HbA$_{1c}$ level and diabetic nephropathy has been challenged by Krolewski *et al.* (30), who have suggested an HbA$_{1c}$ threshold value of approximately 8%, below which the risk for complications is substantially reduced. The apparent benefit of intensified insulin treatment against diabetic nephropathy is supported by the observation that any improvement in HbA$_{1c}$ retards the progression of morphological renal changes (31). It is noteworthy that structural changes in diabetic nephropathy in the kidney have even been completely reversed after 10 years of normoglycemia following pancreas transplantation (32).

With insulin treatment alone, BP increases by 6 mmHg per year in overt nephropathy (12). Effective antihypertensive treatment (AHT) delays the progression of diabetic nephropathy (33-35). Angiotensin-converting enzyme (ACE) inhibitors have a renal protective effect (36-40) which appears in part to be independent of its antihypertensive effect (41). Although thus far no prospective data exist for type 1 diabetes, it is likely that the effect of angiotensin II type 1 receptor inhibitors is similar to that of ACE inhibitors, as shown in large trials in type 2 diabetic patients (42-44).

For type 2 diabetes, evidence indicates that lipid-lowering therapy reduces the level of microalbuminuria (45-47), although these results are not consistent (48). Although prospective data are lacking, it is, however, also likely that type 1 diabetic patients will benefit from such therapy, since an association with dyslipidemia already appears during the microalbuminuric stage, an association even more pronounced in overt nephropathy (49).

Experimental animal models suggest that low dietary protein intake retards the progression of renal disease (50). Although protein restriction is clearly beneficial in non-diabetic renal disease (51), and although it is frequently employed in diabetic patients, evidence for an alleviating effect in diabetic nephropathy is still rather scarce (52-54). Similarly, cessation of smoking is also likely to slow the development and progression of diabetic nephropathy despite a scarcity of evidence for this (55-57).

The most radical improvement regarding the prognosis of the type 1 diabetic patient with nephropathy has been renal replacement therapy. Consequently, diabetes is today the most common diagnosis calling for dialysis or renal transplantation, whereas patients back in the 1970s were still considered too ill for these treatments (58). Although uremia rarely leads to death to-

day, life expectancy after initiation of renal replacement therapy is scarcely more than 5 to 7 years, because of the high comorbidity with cardiovascular disease (28).

In summary, active therapy both before and after renal replacement therapy has resulted in an improvement in median survival time after onset of diabetic nephropathy from 7 years in the 1970s to 14 years now (59). In patients with diabetic nephropathy, prognosis is, however, still poor, and since the incidence of type 1 diabetes is escalating, the number of patients with type 1 diabetes and nephropathy will increase. Focus shall therefore be on preventative measures to avoid not only type 1 diabetes, but also diabetic nephropathy. For its prevention, novel strategies and treatments are needed. These are dependent on novel insights into the pathogenesis of disease initiation and progression. Only accurate understanding of the mechanisms leading to diabetic nephropathy can help us develop specific treatment strategies and procedures.

2.2.4. Changes in renal structure

Kidney volume is already increased at diagnosis of diabetes (60) and is most likely caused by expansion of tubular tissue (61). This probably explains why in diabetes—in contrast to other chronic renal diseases where the size is consid-erably diminished—kidney size is, in spite of ESRD, almost normal. The volume of the individual glomerulus is also enlarged at diabetes diagnosis, and early enlargement is probably due to enhanced collagen production in the basement membrane, whereas later enlargement is probably caused by mesangial expansion and continues to increase later in the disease (62). Thickening of the glomerular basement membrane (GBM) is perhaps the most omnipresent initial lesion of diabetic nephropathy (63) along with the mesangial expansion which appears to occur later (64). The most specific and pathognomonic lesions of diabetic nephropathy, albeit not always present, are the Kimmelstiel-Wilson nodules (65). Hyaline deposits are also specific for diabetic nephropathy: In the afferent and efferent arterioles they can be deposited between the endothelial cell and the basement membrane as "fibrin caps," or finally as "capsular drops" inside Bowman's capsule (66).

During normal UAER or mild micro-albuminuria (UAER 20-30 μg/min), usually no consistent glomerular abnormalities appear. With UAER increase comes an increase in thickness of the GBM and in fractional volume of the mesangium, simultaneously with a minor reduction in creatinine clearance and a rise in arterial BP (67,68). Although UAER correlates with the severity of glomerular lesions, considerable inter-individual overlap occurs (68,69).

2.3. Pathogenesis of diabetic nephropathy

2.3.1. Environmental factors

Numerous environmental factors can increase risk for diabetic nephropathy. Some are poorly documented and observable only in association studies. **Table 1** summarizes environmental and genetic risk factors for diabetic nephropathy based on human studies.

2.3.1.1. Hyperglycemia

Supraphysiological blood glucose levels over decades are necessary but not sufficient to trigger the sequence of events leading to diabetic nephropathy, since only a subset of patients will eventually develop this complication. Hyperglycemia has been demonstrated to induce vasodilatation and basement membrane thickening, as well as apoptosis of endothelial cells *in vitro* (70-72). High glucose concentrations lead to an increased synthesis of extra-cellular matrix from mesangial cells, making them vulnerable to vasoconstrictors and resulting in hyperfiltration (73,74). If normal kidneys of rats and humans are transplanted into a hyperglycemic milieu, they develop lesions typical of diabetic nephropathy (75,76). On the other hand, if normoglycemia can be permanently restored, glomerular lesions typical for diabetic nephropathy can disappear (32).

What are the mechanisms through which hyperglycemia exerts its harmful effect? During sustained hyperglycemia, intermediate glycated Amadori-products form. These are further transformed into irreversible advanced glycation end-products (AGEs). Amadori-albumin has been proposed to cause microvascular complications (77-79). Accumulation of advanced glycation end-products (AGEs) into tissue will interfere with long-lived extracellular proteins, a process which can then influence matrix production, thickening of basement membrane, and binding of heparan sulfate proteoglycans (80). AGEs are increased during hyperglycemia and, as shown in animal studies (81,82), can induce glomerulosclerosis and albuminuria. The amount of AGEs also correlates with diabetic nephropathy and retinopathy (83), and AGEs can predict changes in renal morphology of type 1 diabetic patients (84). Interestingly, if aminoguanidine, which is an AGE inhibitor, is administered to experimental animals with diabetes, the increase in mesangial volume is inhibited and a decrease in UAER occurs (82,85,86). Currently no data are available on aminoguanidine in humans, due to its toxicity.

AGEs can interact with specific receptors, of which the best characterized is the receptor for AGEs (RAGE). This

Table 1. Risk factors and markers for diabetic nephropathy ranked primarily according to evidence of causality and secondarily to association studies in human type 1 diabetic patients

Risk factor/marker	Causality	Association	Available or potential treatment	References
Microalbuminuria	Strong	Strong	ACE inhibitor	(17-19,285)
Glycemic control	Strong	Strong	Improved glycemic control	(29,346)
Upper range normal UAER	Evident	Strong	Blood pressure control	(113,347)
Hyperfiltration	Evident	Evident	Improved glycemic control	(14,108,348)
Elevated blood pressure	Uncertain	Strong	Antihypertensive treatment	(21,22,113-115)
Increase in GBM thickness	Uncertain	Strong	Improved glycemic control	(68,69,349)
Mesangial expansion	Uncertain	Strong	Improved glycemic control	(68,69,349)
Dyslipidemia	Uncertain	Strong	Statins, fibrates	(49,149,154)
Familiar clustering	Uncertain	Strong	NA	(5,192,194,195)
Male sex	Uncertain	Evident	NA	(3,217)
Early onset of diabetes	Uncertain	Evident	NA	(6,112)
Smoking	Uncertain	Evident	Cessation of smoking	(350-352)
Insulin resistance	Uncertain	Evident	PPARγ-agonist	(353-355)
Ethnicity	Uncertain	Evident	NA	(215,356)
Parental hypertension	Uncertain	Evident	NA	(117,118)
Parental CVD	Uncertain	Evident	NA	(205-207)
Low HSPG in GBM	Uncertain	Evident	NA	(176,357,358)
ACE I/D polymorphism	Uncertain	Evident	NA	(226,359,360)
High prorenin and renin	Uncertain	Evident	NA	(229,361)
High protein intake	Uncertain	Evident	Protein restriction	(51)
Na+/Li+ countertransport ↑	Uncertain	Uncertain	NA	(362)
Parental type 2 diabetes	Uncertain	Uncertain	NA	(202)
Endothelial dysfunction	NK	Evident	Improved glycemic control	(363)
AGEs	NK	Evident	Aminoguanide	(364)
High TGFβ levels	NK	Evident	TGFβ inhibitors	(365,366)
High PKC activity	NK	Evident	PKC inhibitors	(367)
Short stature	NK	Uncertain	NA	(163,167)
Low birth weight	NK	Uncertain	NA	(164)
No C-peptide production	NK	Uncertain	C-peptide	(368)
Na+/H+ exchanger activity↑	NK	Uncertain	NA	(369,370)
High iron deposits	NK	Uncertain	Reduced iron intake	(371)
Hyperandrogenicity	NK	Uncertain	Testosterone antagonists	(372,373)
Lack of physical exercise	NK	Uncertain	Optimal amount of exercise	(374)
High VEGF	NK	Uncertain	NA	(375)
High GH and IGF-1 levels	NK	Uncertain	Somatostatin analogue	(142)
Overactive polyol pathway	NK	Uncertain	Aldose-reductase inhibitor	(376)
Reduced number of glomeruli	NK	Uncertain	NA	(377)
Proliferative retinopathy	Absent	Strong	Retinal laser treatment	(29)
Neuropathy	Absent	Evident	NA	(29)

NA = not available, NK = not known. For other abbreviations, see page 7.

receptor is expressed in renal interstitial cells and vascular smooth muscle cells (87); in experimental studies, AGE-RAGE interaction has been shown to induce oxidative stress (88). The co-localization of RAGE in renal glomeruli suggests that this AGE-RAGE interaction may represent an important mechanism in the pathogenesis of diabetic complications (89). Interestingly, over-expressed human RAGE in diabetic transgenic mice induces glomerular hypertrophy, increased albuminuria, mesangial expansion, advanced glomerulosclerosis, and an increase in serum creatinine none of which is evident in mice lacking the *RAGE* transgene (90). Moreover, a significant upregulation of RAGE occurs in human vascular disease (91). By blocking the AGE/RAGE binding, the cellular changes associated with formation of atherosclerosis in animal models of diabetes are prevented (92).

Hyperglycemia can also influence other biochemical pathways. Aldose reductase is the first and rate-limiting enzyme of the polyol pathway in which glucose is transformed into sorbitol. An intracellular accumulation of sorbitol occurs during hyperglycemia and, along with disturbances in cellular osmoregulation, results in tissue damage (93). These metabolic abnormalities may be involved in the pathogenesis of diabetic nephropathy (94). Unfortunately, treatment with tolrestat, an aldose reductase inhibitor, has been disappointing in humans (95,96), although rats showed a reduction in UAER (97,98).

Another common pathway suggested to mediate the detrimental effects of hyperglycemia is the activation of protein kinase C (PKC). Activation of PKC stimulates extracellular matrix production, expression of growth factors, and contractility, permeability, and vascular cell proliferation (99). Although no data exist on inhibition in humans of the PKCβ isoform, such inhibition in rats prevents an increase in cytokine expression, reverses abnormal retinal and renal hemodynamics, and also reduces UAER (100).

It is also possible that glucose itself has toxic effects on the cell. If cultured human endothelial cells are chronically exposed to high glucose concentrations, they show abnormalities not due to an altered polyol pathway (101). Chronic hyperglycemia also results in increased expression and synthesis of collagen, fibronectin, and laminin (102), as well as a decrease in heparan sulphate by mesangial cells (103). High glucose levels in mesangial cells also induce the transcription and secretion of transforming growth factor beta (TGFβ) which stimulates matrix synthesis and inhibits its degradation (104).

2.3.1.2. Hemodynamic factors

During hyperglycemia an increase occurs in cardiac output and peripheral vasodilatation (105,106) which results in disturbed autoregulation of the blood flow in several organs and perhaps in elevated BP in capillaries of several end-organs (107). It has been suggested that glomerular hypertension and hyperfiltration are key players in the pathogenesis of diabetic nephropathy (108), since elevated intraglomerular pressure can alter the milieu within the glomerulus and lead to an increase in protein filtration and protein accumulation in the mesangium, as well as mesangial expansion (108). Such an expansion may cause stretching of mesangial cells, increased extracellular matrix production, glomerular hypertrophy, and tubulointerstitial inflammation (109).

Several animal models have shown that BP control can prevent glomerulosclerosis (110,111). In humans, elevated arterial BP is an early phenomenon (22,112), but whether it also precedes a rise in UAER is debatable (113-116). Evidence indeed exists for a parallel rise in UAER and BP, as shown in a longitudinal study using 24-hour ambulatory BP monitoring in which baseline BP did not differ between patients progressing to microalbuminuria and non-progressors (21). If this is true, the increase in blood pressure could be considered a secondary phenomenon and due to the renal dis-ease. However, we have shown (117), and also have confirmed earlier data (118) that an elevated BP may even be a primary phenomenon and may precede the onset of renal disease, since BP is significantly higher in parents of those type 1 diabetic patients who develop diabetic nephropathy than of those that preserve a normal UAER. Such a view supports the hypothesis that susceptibility to hypertension may also represent susceptibility to diabetic nephropathy.

It is thus possible that common pathogenetic mechanisms exist for the elevated BP seen in non-diabetic subjects and the initiation or progression of diabetic nephropathy in type 1 diabetic patients. Data are convincing that blocking the angiotensin-converting enzyme (ACE) of the renin-angiotensin-aldosterone system (RAAS) lowers BP and postpones development of diabetic nephropathy (41,119,120). A similar blood-pressure lowering as well as renoprotective effect is evident when the angiotensin II type 1 receptor (AT1) is blocked in type 2 diabetic patients with diabetic nephropathy (42-44). It is of note that in non-diabetic subjects the angiotensin II type 2 receptor (AT2) seems to oppose the deleterious effects of the AT1 through vasodilatation and a subsequent decrease in blood pressure (121). AT2 stimulation also confers renal protection in rats with progressive renal injury (122), and is present in the rat kidney in glomerular epithelial cells,

cortical tubules, and interstitial cells (123). Data regarding the impact of the AT2 on diabetic nephropathy are scarce, although alterations in the balance of kidney AT1 and AT2 receptor expression have been reported to contribute to the glomerular injury mediated by angiotensin II in progressive diabetic nephropathy (124).

Another important renal hemodynamic modulator is the dopamine system. Dopamine is synthesized independently of nervous activity in the kidney and it acts through at least five receptors, all of which are expressed in the kidney (125). An increase in renal dopamine synthesis and stimulation of vascular dopamine D1 receptors in diabetic rats appears to prevent early glomerular hyperfiltration (126). The synthesis and secretion of renal dopamine have been reported to be lower in diabetic nephropathy in type 2 diabetic patients (127). Interestingly, disruption of the dopamine D3 receptor (DRD3) results in a renin-dependent form of hypertension (128), although this receptor has not yet been studied in diabetic nephropathy.

Neuropeptide Y (NPY) is a vasomotoric polypeptide shown in experimental studies to enhance basal renal blood flow and to alter renal renin levels (129). Stimulation of the NPY receptors has resulted in an increase in sodium excretion and a decrease in glomerular filtration rate, aldosterone concentration, and plasma renin activity (130). NPY also reduces the contractile response to noradrenaline in rats, an effect enhanced by hyperglycemia (131). In addition to its role as a hemodynamic modulator, NPY is an important regulator of energy homeostasis (132) and is associated with low-density lipoprotein (LDL) cholesterol levels in non-diabetic subjects (133). Data are rather scarce regarding diabetic complications and NPY, although both NPY and its Y2 receptor have been associated with diabetic retinopathy in type 1, as well as in type 2 diabetic patients (134,135).

2.3.1.3. Cytokines, growth factors, and inflammation

Cytokines are peptides that are key players in tissue formation, cell differentiation, and tissue repair. Several cytokines are active in synthesis and degradation of the GBM. Hyperglycemia induces an increased expression of TGFβ in rats, with subsequent renal hypertrophy and increased formation of extracellular matrix (136,137). During hyperglycemia in diabetic nephropathy there also occurs an increased synthesis and expression of TGFβ, leading to cyclic stretching of mesangial cells (138) and to progressive matrix accumulation and tissue fibrosis (139).

Among other growth factors, growth hormone (GH) and insulin-like growth factor (IGF-1) have been subject to much

interest in regard to diabetic nephropathy. GH and IGF-1 knock-out mice have less glomerular hypertrophy and a lower UAER than do GH/IGF-I intact rats (140). When a GH antagonist gene is transferred to mice, it causes also less glomerular hypertrophy and a decreased urinary protein content (141). Interestingly, human type 1 diabetic patients show an association between urinary excretion of IGF-1 and renal volume as well as between the excretion of these two growth factors, GH and IGF-1, and microalbuminuria (142).

Several other factors may prove of interest in diabetic nephropathy, but thus far only sparse information is available regarding these substances in type 1 diabetes. Angiogenesis is induced by vascular endothelial growth factor (VEGF), a growth factor that also raises vascular endothelial permeability in the kidney (143). VEGF is also expressed by the glomerular epithelial cell (143). The cytokine interleukin-1 (IL-1) stimulates mesangial cell proliferation (144), and in diabetic rats, glomerular hyperperfusion affects secretion of IL-1 beta. Interestingly, the low-grade inflammatory markers IL-6 and hypersensitive C-reactive protein (145), as well as mannose-binding lectin (146,147), a component of the complement system, have also been associated with diabetic nephropathy in type 1 diabetes. Angiotensin II, a strong vasoconstrictor, induces glomerulosclerosis independently of its hemodynamic

effect, and can also lead to diminished matrix degradation (148).

2.3.1.4. Lipids

A typical feature of diabetic nephropathy is hyperlipidemia (49). Whether hyperlipidemia causes glomerular injury remains unknown, although lipid abnormalities are already present during microalbuminuria (49,149,150). Serum triglyceride level is an independent risk factor for diabetic nephropathy in type 1 (151) and type 2 diabetes (152). Lipid-lowering treatment in almost totally nephrectomized rats has been associated with a small reduction in proteinuria and glomerulosclerosis without any adverse effects on renal function and plasma lipid levels (153,154). In overt nephropathy, hypercholesterolemia has been associated with cardiovascular mortality (155), and with a rapid decline in renal function (155-157). Evidence also exists that lovastatin treatment improves renal function in rats (158), albeit no effect was evident in patients with type 1 diabetes (159).

2.3.1.5. Short stature and low birth weight

Short stature has been proposed as a cardiovascular risk factor in non-diabetic subjects (160-162). In diabetic patients,

two Danish studies showed that males with overt nephropathy were significantly shorter than males with microalbuminuria or normal UAER (163,164). The mechanism is unknown, although an adverse intrauterine environment (165), hyperglycemia, parental social class (166,167), and genetic factors (166) have been suggested.

The Barker hypothesis suggests an association between low birth weight and cardiovascular mortality (168), hypertension (169,170), BP (171,172), and other features of the insulin resistance syndrome (173). The notion that this is caused by environmental factors is supported by the observation that the smaller twin of a genetically identical monozygotic twin pair has the higher BP later in life. Intrauterine environmental factors may thus affect BP differently despite a common genetic background (174). Our own studies have shown that birth weight is inversely correlated with systolic BP and pulse pressure (PP), although its relation to diabetic nephropathy remains to be established (175).

2.3.1.6. Glomerular capillary wall

Endothelial dysfunction reflects a generalized vascular process affecting the glomeruli and large vessels. According to the Steno hypothesis, the barrier between capillary and urinary space loses its negative charge selectivity due to the loss of heparan sulfate proteoglycan (HSPG) content (176). This results in an increase in leakage of albumin through the capillary wall and may thus be one initiating mechanism behind microalbuminuria. A more recent study has confirmed in type 1 diabetes the reduction in HSPG in the GBM, although that study provided no evidence for its involvement with early microalbuminuria (177).

The glomerular podocyte layer has drawn much recent attention with the discovery—in the Finnish type of congenital nephrosis—that the nephrin protein is absent, with a subsequent lack of slit diaphragm and foot processes plus massive proteinuria already in uteri (178). Adult type 1 diabetic patients show down-regulation of nephrin mRNA and a loss of the electron dense structure of the slit diaphragm, thereby suggesting a novel mechanism for diabetic nephropathy (179). Interestingly, a reduction in expression of the nephrin gene and protein can be attenuated by an AT1 blocker (180,181) or an ACE inhibitor (182,183). Nephrin has also been connected in the diabetic mouse with early changes in diabetic nephropathy (184), and downregulation of the nephrin protein may be due to glycated albumin and angiotensin II (185). Recent studies suggest, however, that other proteins integrated with nephrin, such as the CD2-associated protein and podocin, may together contribute to the pathogenesis of proteinuria (186). Leakage of

nephrin into the urine has also recently been suggested to be an early marker of diabetic nephropathy (187).

2.3.2. Genetic factors

Today, substantial evidence exists for the role of genetic factors in the pathogenesis of diabetic nephropathy, although the inheritance pattern is not as yet known. A plausible genetic model may be one or two major genes with a dominant or recessive effect interacting with environmental factors, or a few genes with a moderate effect in a similar interaction (188). Diabetic nephropathy thus may represent a typical complex disease such as type 1 diabetes plus asthma. The proportion of the genetic involvement in a complex disease is often expressed as sibling recurrence risk, or lambda S (λ_S), which is the risk of a sibling's developing the disease which the other sibling already has. As can be calculated from the study of Quinn *et al.* (5), λ_S in diabetic nephropathy is approximately 2 to 2.5, lower than that calculated for type 1 diabetes ($\lambda_S = 15$) (189) or for asthma ($\lambda_S = 3$-5) (190).

2.3.2.1. Incidence studies

In diabetic nephropathy, incidence rate peaks about 15 years after diabetes diagnosis, after which it declines substantially (3,4,6,191), in sharp contrast to diabetic retinopathy, where no such peak is apparent and incidence rate is linear. This observation can be interpreted to imply that some genes either expose the subject to or protect the subject from diabetic nephropathy during a certain time-span (188). If, after 25 years, a patient with diabetes still has a normal UAER, the lifetime risk of developing overt nephropathy and ESRD is small.

2.3.2.2. Familial clustering and heritability

Familial clustering of diabetic nephropathy has provided strong evidence for genetic involvement. The original observation by Seaquist *et al.* (192) showed elevated UAER in 83% of diabetic siblings of patients with diabetic nephropathy, whereas among siblings with a normal UAER, only 17% had nephropathy. Although these original figures were criticized as genetically implausible (193), three later studies have confirmed this finding, despite the fact that their degree of familial clustering seems to be considerably lower (5,194,195). Although such figures may be due to sharing of the same environment, simulation studies have shown that environmental factors cannot entirely account for the familial aggregation of such a trait (196).

The genetic model of diabetic nephropathy is still unclear, partly due to the

fact that no available data exist from twin studies, in contrast to such conditions as type 1 diabetes, in which the concordance rate has been 27% in monozygotic and 4% in dizygotic twins (197). However, in diabetic nephropathy of type 2 diabetic patients, a single major locus or a dominant model with additional multifactorial effects is proposed, based on recent segregation analyses (198,199). It is noteworthy, in contrast, that one large study reported familial clustering for retinopathy but not for nephropathy in type 1 diabetes, although this lack of correlation may have been due to the short duration of diabetes in the diabetic offspring (200). On the other hand, there appears to be strong concordance between severity and patterns of glomerular lesions in type 1 diabetic siblings despite their simultaneous lack of concordance for glycemia, which supports a theory of genetic factors' affecting diabetic nephropathy (201).

There also appears to be familial clustering of certain risk factors in diabetic nephropathy. We and others have discovered more hypertension (117,118), type 2 diabetes (202), and insulin resistance (203) in parents of patients with type 1 diabetes and overt nephropathy than in those with normal UAER. Similarly, parents of patients with diabetic nephropathy also have higher mortality and more CVD, although these findings are not consistent (202,204-207). Nondiabetic first-degree relatives of type

2 diabetic patients with diabetic nephropathy seem to show have an elevated UAER in comparison to those with normal UAER (208) (209-211), although this may not be the case in type 1 diabetes (212).

2.3.2.3. Ethnic variation

Substantial differences in prevalence of diabetic nephropathy have emerged between various ethnic groups. African-Americans have a 5-fold increased risk for developing ESRD compared to Caucasian type 2 diabetic patients, which cannot be explained by differences in glycemic control or in diabetes duration (213). This increased risk in African-Americans may, however, be a marker of an inherited susceptibility to development of renal disease, rather than to development of diabetic nephropathy. Similarly, incidence of diabetic ESRD in Native Americans is 7-fold higher than in the Caucasian population (214). Among Jews with type 1 diabetes, the non-Ashkenazis are at higher risk for nephropathy than are other Jewish groups (215).

2.3.2.4. The role of gender

Due to the paucity of large prospective studies, the role of gender is not entirely clear. Because of the excess cardiovas-

cular mortality in both diabetic and non-diabetic males (216), results of prevalence studies may be biased, undermining any potential difference between males and females. Evidence does exist that in males both the prevalence of diabetic nephropathy is higher (3) and the progression of ESRD is faster (3,217). This male preponderance is further supported by an 18-year follow-up study from Denmark in which male gender was an independent predictor for microalbuminuria (218).

2.4. Genetic approaches to complex traits and diabetic nephropathy

A complex genetic trait implies that the phenotype is not the result of classic Mendelian recessive or dominant inheritance caused by a variation in a single gene at a single locus. Since the cumulative incidence of diabetic nephropathy is approximately 30%, it is likely that several genes each play only a modest role in the disease process. The two major approaches in the search for susceptibility genes for complex traits are linkage analysis and association studies, the latter often referred to as candidate gene studies.

2.4.1. Linkage analysis

Traditional linkage analysis requires that certain estimates must be provided for all variables for the traits studied, including mode of inheritance, penetrance, phenocopies, and gene frequencies (219). This approach usually requires a rather high number of sibling pairs or larger pedigrees. Regarding sibling pairs, an allele-sharing method can be used requiring no disease model variables, since it relies on affected relatives, each sharing a different proportion of alleles than would occur randomly.

In a genome-wide scan, markers randomly spread over the entire genome cover all chromosomes. If one marker is inherited from each parent more often than predicted, linkage has occurred, and consequently, this region may harbor a susceptibility gene. This method requires no previous knowledge of the putative gene. In diabetic nephropathy in type 1 diabetes, currently no genome-wide scans are published, and the number of linkage studies is also small. One such linkage study concentrated on markers at three loci in the RAAS (220), and found significant linkage to chromosome 3q in the region harboring the angiotensin II type 1 receptor gene (*AT1*). However,

although the gene was not entirely covered in that study due to very extensive intronic regions, no variants of the *AT1* gene showed any association with diabetic nephropathy. What cannot be ruled out is whether a nearby unknown gene could have been responsible for the linkage observed. The locus on 3q23-24 is now replicated in a very recent Russian study (221). Regarding diabetic nephropathy in type 2 diabetic patients, thus far only one genome-wide scan has appeared, and that study found linkage on chromosomes 7, 3, 9, and 20 (222).

2.4.2. Association studies

This method requires identifying genetic variations or polymorphisms in a candidate gene that may alter the coding sequence or transcription activity and may thus alter the function or expression of proteins involved in the disease process. The design most frequently used is a case-control study. The frequency of gene variation in subjects with a disease (cases) is compared to the frequency of those without disease (controls). This approach suffers substantially, however, from both false positive (type I error) and false negative results (type II errors), highlighted by its rather low reproducibility. This may be due to a spurious association with a number of causes such as genetic heterogeneity or the presence of phenocopies. Appropriate and careful characterized cases and controls are thus needed.

In order to overcome these problems, alternative designs have been developed. In nuclear family or trio designs, the transmission disequilibrium test (TDT) requires no control group, since the genotypes of the patient's parents are known (223). Such a family-based study scrutinizes how the putative disease allele is transmitted to the patients from their heterozygous parents. If it is transmitted in significantly more often than in half of the cases, the disease allele is associated with the trait.

2.5. Candidate gene studies of diabetic nephropathy in patients with type 1 diabetes

As mentioned, there are no published genome-wide scans in diabetic nephropathy in type 1 diabetic patients, due to the difficulty in enrolling a sufficient number of sibling pairs concordant or discordant for diabetic nephropathy, partly since there is a long time-span between onset of diabetes and ascertainment of nephropathy status. In addition, patients with type 1 diabetes and ESRD have high mortality rates, and of course incur rather high expense for a genome-wide scan. Studies on the genetics of diabetic nephropathy in type 1 diabetes have therefore been almost entirely association studies. Due to the several factors

suggested to be involved in the pathogenesis, numerous candidate genes have already been studied in patients with diabetic nephropathy. These have frequently included genes that have previously been studied in hypertension, type 2 diabetes, and cardiovascular disease (224). None of the associations of gene variants with diabetic nephropathy found have been convincingly and undisputedly replicated in other populations or in prospective studies.

2.5.1. The renin-angiotensin-aldosterone system

The genes of the RAAS have drawn special attention in diabetic nephropathy, since it is well known that ACE inhibitors effectively reduce proteinuria beyond the reduction in BP levels (41).

Consequently, the *ACE* insertion/deletion (I/D) polymorphism is the polymorphism most studied. A total of 27 publications during the last 10 years deal with the *ACE* I/D polymorphism; these show rather high variability. Two meta-analyses have indicated a weak association for the D-allele as a risk marker for diabetic nephropathy in which the odds ratio (95% confidential interval) was 1.12 (0.95-1.32) (225,226). **Table 2** summarizes published candidate gene studies on the *ACE* I/D as well as on other genes of the RAAS in diabetic nephropathy in type 1 diabetes.

Another important gene studied in the RAAS is the angiotensinogen gene. A positive association appeared for the M235T polymorphism in a TDT study in male subjects (227). Otherwise, results also conflict in regard to this polymorphism (**Table 2)**.

Table 2. Candidate genes in the RAAS studied in type 1 diabetic patients with nephropathy

Gene	Polymorphism	Positive association Reference	No association Reference
ACE	I/D	(225,226,306,359,360,378-385)	(296,386-398)
ACE	*Pst*I	(297)	
Angiotensinogen (*AGT*)	M235T	(227,398,399)	(359,387,391,395,397,400,401)
AT1	A1166C		(220,397,398,402-405)
AT1	T573C	(406)	
Bradykinin 1 receptor	G-699C		(228)
Renin	*Bgl*I	(229)	

The angiotensin II type 1 receptor gene (*AT1*) has been the focus of several studies, partly because of the study by Moczulski *et al.* (220), showing a strong linkage in the region containing this gene. However, association studies have mainly been negative for the *AT1* gene. Other genes of the RAAS studied comprise the bradykinin-1 receptor, not associated with nephropathy in a Caucasian cohort of both type 1 and type 2 diabetic patients (228). Interestingly, in a Danish cohort, a positive association appeared between a polymorphism in the renin gene and diabetic nephropathy (229). Unfortunately, this finding has not been replicated in other populations. Despite interest in the angiotensin II type 2 receptor (AT2) as one of the key players in the RAAS, the corresponding gene (*AT2*) has as yet not been studied in diabetic nephropathy.

2.5.2. Genes related to blood pressure

Since, in patients with diabetic nephropathy, elevated BP is a characteristic feature, genes involved in BP regulation have drawn much attention, as summarized in **Table 3**. One interesting question is whether the pronatriodilatin gene, coding the atrial natriuretic factor (ANP), is involved in diabetic nephropathy.

Table 3. Candidate genes related to blood pressure studied in type 1 diabetic patients with nephropathy

Gene	Polymorphism	Positive association Reference	No association Reference
Alpha adducin	Gly460Trp		(236)
ATP1 A1	*Bg*l II		(407)
Beta3 adrenergic receptor	Trp64Arg		(408)
Epithelial sodium channel	BetaArg564X		(409)
G-protein beta 3	C825T	(410)	(411,412)
Nitric oxide 3 (e*NOS*)	4a/b	(233)	(234,235)
Nitric oxide 2A	CCTTT-repeat	(413)	
Nitric oxide 2A	promoter		(235)
Pronatriodilatin (*ANP*)	*Sca*l	(230)	
Pronatriodilatin (*ANP*)	T2238C	(231)	
Pronatriodilatin (*ANP*)	*Hpa*II (intron 2)		(232)
SA hypertension-associated homologue	*Pst*1		(414)

Thus far, results have been rather conflicting (230-232). Given the substantially large variation between these studies, it could be argued that this discrepancy is due to ethnic differences. A positive association appeared between the endothelial nitric oxide gene (e*NOS*) and advanced diabetic nephropathy in an American cohort examined by both case-control and family design (233-235). Although the alpha-adducin gene has been associated with essential hypertension, in a type 1 diabetic cohort from Ireland and Northern Ireland no association existed between this gene and diabetic nephropathy or hypertension (236).

Although the dopamine system regulates BP (125), and although all dopamine receptor genes (*DRD1-DRD5*) have been characterized, no studies thus far have explored diabetic complications.

One potential gene in this family is the *DRD3*, since disruption of this gene in mice results in a renin-dependent form of hypertension (128).

2.5.3. Genes related to cardiovascular disease

Due to the high prevalence in diabetic nephropathy of early atherosclerosis and cardiovascular complications, genes associated with CVD have frequently been studied as candidate genes for nephropathy, as shown in **Table 4**. The C677T polymorphism of the methylenetetrahydrofolate reductase (*MTHR*) gene is associated with plasma homocystein levels, which may contribute to cardiovascular disease (237). This polymorphism has been studied by

Table 4. Candidate genes related to cardiovascular disease studied in type 1 diabetic patients with nephropathy

Gene	Polymorphism	Positive association Reference	No association Reference
Apolipoprotein E	e2,e3,e4	(243,244)	(245,246)
Beta3 integrin	PlA1/PlA2		(415)
Beta-fibrinogen	G455A		(416)
Methylenetetrahydrofolate reductase	C677T	(238,242)	(239-241)
Plasminogen activator inhibitor-1	4G/5G		(390,417)
Paraoxonase 1 (*PON1*)	Several		(418)
Paraoxonase 2 (*PON2*)	Several		(419)
Werner syndrome helicase	C/R		(390)
von Willebrand factor	T789A, A138T		(420)

several groups in regards to diabetic nephropathy, but the results are again conflicting (238-242). Prospective studies are needed to further elucidate the role of this gene. A quite similar constellation exists for the e2, e3, and e4 alleles of the apolipoprotein E gene, with two positive (243,244) and two negative studies (245,246).

Interestingly, a functional Leu7Pro polymorphism of the preproneuropeptide Y has been associated with carotid atherosclerosis, serum LDL cholesterol levels, triglycerides, and BP in Finnish non-diabetic subjects (133,247,248).

The Leu7Pro has also been associated with both retinopathy and carotid atherosclerosis in Finnish type 2 diabetic patients (135,249). Its role in late complications in type 1 diabetic patients has, however, not yet been elucidated.

2.5.4. Genes related to diabetes, immunology, and glucose metabolism

The first candidate gene study involving patients with diabetic nephropathy was published in 1991. Raffel *et al*. (250)

Table 5. Candidate genes related to diabetes, immunology, and glucose metabolism studied in type 1 diabetic patients with nephropathy

Gene	Polymorphism	Positive association Reference	No association Reference
Genes related to diabetes and immunology			
Complement C4	C4AQ0		(421)
ENPP1/PC-1	K121Q		(380)
Human leucocyte antigen	Several		(251)
Hepatocyte nuclear factor 1 b	E260D,Q378Q		(422)
Insulin	Ins+/-		(251)
Insulin	5'end	(250)	
Genes involved in glucose metabolism			
AGE-receptor 1	Several		(255)
AGE-receptor 2	Several		(255)
AGE-receptor 3	Several		(255)
AGE-receptor (*RAGE*)	Several		(255)
Aldose reductase	(CA)n	(220,252,423,424)	(254,425,426)
GLUT1	*Xba*I	(427,428)	(429)

found a positive association between a polymorphism in the 5'end of the insulin gene, a finding not yet consistently replicated (251).

A few other studies dealing with genes related to diabetes and glucose metabolism have been performed, as shown in **Table 5**. Regarding the aldose reductase gene (*AL2*), a group from the UK found a strong association in Caucasian patients between a (CA)n repeat and diabetic nephropathy ($\chi^2 = 18.6$, $P < 0.00001$) (252). Interestingly, this positive association was later repeated in an American cohort (253), but not in another cohort of Caucasian patients from the UK (254). Poirot *et al.* (255) detected a positive association between longer duration of nephropathy-free diabetes

and the C-1152A polymorphism of the *RAGE* gene promoter, an association later complicated by discovery of a pseudogene adjacent to the *RAGE* promoter (256). No replication study dealing with this interesting gene has appeared.

2.5.5. Genes related to glomerular structure

Although the basic structure of the glomerulus and its proteins are well characterized, the genes regulating these proteins and glomerular function are rather poorly known. Only a few candidate genes have been studied regarding structural proteins, as seen in **Table 6**.

Table 6. Candidate genes related to glomerular structure studied in type 1 diabetic patients with nephropathy

Gene	Polymorphism	Positive association Reference	No association Reference
Caldesmon	-579A>G	(260)	
Collagen IVA1	*Hind*III		(430)
Decorin	179/183/185	(258)	
Heparan sulphate proteoglycan	*Bam*HI	(257)	

A study in a combined Danish-British cohort with respective 260 + 397 patients showed a weak positive association between *Bam*HI polymorphism of the heparan sulphate core protein (*HSPG*)

gene and UAER (257). This finding has not been confirmed, although it supports the Steno hypothesis that a defect in the GBM leads to loss of charge selectivity and subsequent proteinuria (176). An

allelic variant of another basement membrane gene, decorin, has been associated in a longitudinal study with a 6.5 year-follow-up with a slower progression of nephropathy (258).

Dysfunction of the glomerular actin cytoskeleton, a component of the glomerular podocyte layer, occurs in diabetic nephropathy (259). Interestingly, a positive association was recently found in two distinct Irish cohorts between diabetic nephropathy and caldesmon, one of the genes coding the actin cytoskeleton, (260). Another important protein, a major component of the glomerular slit membrane called nephrin, was discovered several years ago (178). Several mutations in the nephrin gene (*NPHS1*)

lead to a complete loss of nephrin from the slit diaphragm and cause the Finnish type of congenital nephrosis characterized by massive proteinuria and early death (261). It can, however, be hypothesized that some mutations lead to a minor defect in the protein, instead of a total loss of the protein. In a diabetic milieu, such a defect probably could cause a milder degree of proteinuria.

2.5.6. Other genes

A few other genes mostly dealing with cytokines and growth factor have been scrutinized in diabetic nephropathy, as seen in **Table 7.**

Table 7. Genes related to growth factors and enzymes studied in type 1 diabetic patients with nephropathy

Gene	Polymorphism	Positive association Reference	No association Reference
Interleukin-1A	C/T -889		(262,263)
Interleukin-1B	ex5 T/C	(262)	(263)
Interleukin-1R1	ex1B T/C		(262,263)
Interleukin-1RN	intr2 86 bp rep.		(262,263)
N-acetyltransferase	fast/slow	(431)	
Nuclear factor kappa β	A1-A18		(432)
Transforming growth factor β1	Several		(264)
Transforming growth factor β1	Thr263Ile	(265)	
VEGF	D/I -2549	(266)	

Two groups studying the interleukin system on chromosome 7q35 showed that the IL-1 cluster is not a key player in the development of diabetic nephropathy (262,263), although a peak containing this IL-1 locus appeared in a genome-wide scan in type 2 diabetic patients with nephropathy (222). Several polymorphisms of the $TGF\beta$ gene have been characterized, and two candidate gene studies published regarding this gene and diabetic nephropathy. However, as so often occurs, the results are rather conflicting (264,265). A recent study found a positive association between variants in the $VEGF$ gene and diabetic nephropathy in type 1 diabetic patients from the UK (266). Interestingly, this association was further strengthened when analyzed together with polymorphisms in the aldose reductase gene, suggesting a gene-gene interaction.

3. AIMS OF THE PRESENT STUDY

Diabetic nephropathy is associated with massively increased risk for cardiovascular disease and early mortality. Although only a subset of the patients with type 1 diabetes will develop this devastating complication and evidence is convincing for a genetic impact in its pathogenesis, the predisposing genes have been hitherto unknown. The studies in this thesis were performed to answer the following questions concerning type 1 diabetic patients:

1. Are three common polymorphisms in the receptor for the advanced glycation end-products gene (*RAGE*) involved in pathogenesis of diabetic nephropathy and other late complications? (I)

2. Are three exonic polymorphisms in the nephrin gene (*NPHS1*) associated with proteinuria or renal function? (II)

3. Is the Leu7Pro polymorphism of the preproneuropeptide Y (*NPY*) associated with glycemic control, lipid profile, BP levels or macrovascular complications? (III)

4. Is any polymorphism or haplotype studied in the dopamine D3 receptor gene (*DRD3*) associated with diabetic nephropathy, renal function or BP levels? (IV)

5. Can any of the two polymorphisms or haplotypes studied in the X-chromosomal angiotensin II type 2 receptor gene (*AT2*) explain the male predominance in diabetic nephropathy, or is the gene associated with renal function or BP levels? (V)

4. SUBJECTS AND STUDY DESIGN

4.1. Study population

This study was part of the ongoing Finnish Diabetic Nephropathy (FinnDiane) Study; a comprehensive multicenter, nationwide project launched in November 1997, with the aim of phenotyping 20 to 25% of all adult patients with type 1 diabetes in Finland, and to study diabetic late complications. All patients with type 1 diabetes available in the FinnDiane database in August 1999 were classified according to their UAER, as shown in **Figure 2**. At that time there were 1006 classifiable patients with an ascertained renal status in the FinnDiane database from 20 secondary or tertiary referral centers. All patients were required to be C-peptide-negative (< 0.3 nmol/l), and to have permanent insulin treatment initiated within one year after the diabetes diagnosis and before age 40. Patients with evidence of non-diabetic kidney disease were excluded. Eventually, 996 patients participated.
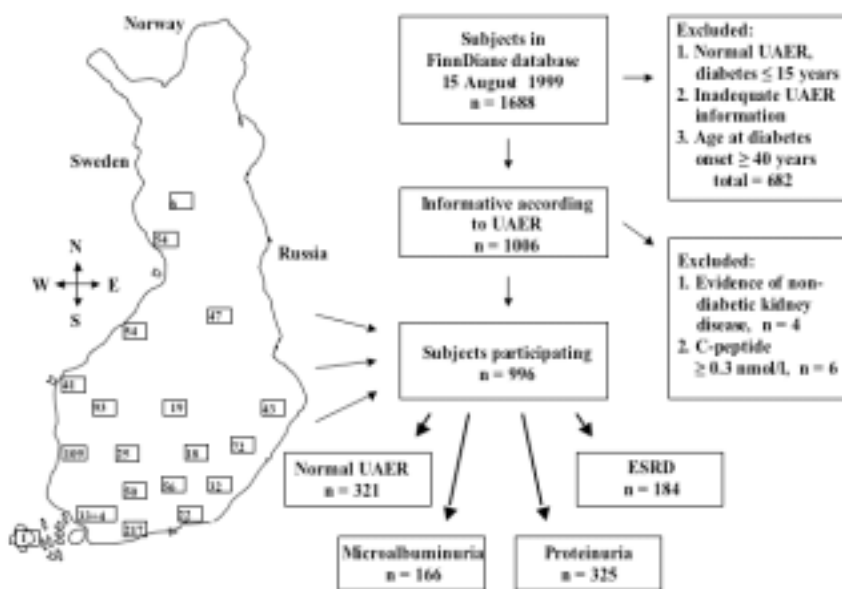

Figure 2. Flow-chart depicting recruitment procedure for study subjects.

Table 8. Clinical characteristics of all 996 study patients

Variable	Data available (%)	All n=996	Normal UAER n=321	Microalbuminuria n=166	Proteinuria n=325	ESRD n=184
M/F (%)	538/458 (100)	54/46	40/60	64/36	60/40	59/41
Age (years)	996 (100)	40.7 ± 0.3	40.4 ± 0.5	38.1 ± 0.9	40.3 ± 0.5	44.0 ± 0.6
Duration of diabetes (years)	996 (100)	27.8 ± 0.3	26.7 ± 0.4	25.1 ± 0.7	28.0 ± 0.4	32.0 ± 0.6
BMI (kg/m²)	973 (98)	25.2 ± 0.1	24.9 ± 0.2	25.7 ± 0.3	25.9 ± 0.2	23.8 ± 0.3
Waist-to-hip ratio male	516 (96)	0.93 ± 0.01	0.90 ± 0.01	0.91 ± 0.01	0.94 ± 0.01	0.95 ± 0.01
female	433 (95)	0.82 ± 0.01	0.81 ± 0.01	0.82 ± 0.01	0.83 ± 0.01	0.87 ± 0.01
Antihypertensive treatment (%)	967 (97)	604 (62)	47 (15)	99 (62)	304 (96)	154 (91)
ACE inhibitors (%)	967 (97)	396 (41)	32 (10)	83 (52)	243 (76)	38 (22)
Cardiovascular disease (%)	987 (99)	106 (11)	10 (3)	6 (4)	42 (13)	48 (27)
Acute myocardial infarction (%)	985 (99)	53 (5)	5 (2)	4 (2)	21 (7)	23 (13)
Coronary heart disease (%)	957 (96)	85 (9)	9 (3)	6 (4)	33 (11)	37 (21)
Stroke (%)	984 (99)	37 (4)	2 (1)	0 (0)	17 (5)	18 (10)
PVD by-pass (%)	985 (99)	41 (4)	1 (0)	2 (1)	18 (6)	20 (11)
Any retinopathy (%)	979 (98)	817 (83)	203 (64)	130 (80)	302 (96)	182 (100)
Retinal laser treatment (%)	985 (99)	615 (62)	92 (29)	84 (51)	260 (81)	179 (99)
Current smokers (%)	970 (97)	220 (23)	52 (17)	43 (26)	94 (29)	31 (18)
SBP (mmHg)	964 (97)	141 ± 1	132 ± 1	138 ± 1	144 ± 1	154 ± 2
DBP (mmHg)	963 (97)	82 ± 1	79 ± 1	82 ± 1	83 ± 1	87 ± 1
Pulse pressure (mmHg)	963 (97)	59 ± 1	53 ± 1	56 ± 1	61 ± 1	67 ± 1
HbA$_{1c}$ (%)	979 (98)	8.5 ± 0.1	8.0 ± 0.1	8.7 ± 0.1	8.9 ± 0.1	8.4 ± 0.1
eGDR (mg · kg⁻¹ · min⁻¹)	919 (92)	5.8 ± 0.1	8.1 ± 0.1	5.7 ± 0.1	4.2 ± 0.1	4.5 ± 0.1
UAER (mg/24h)[a]	582 (58)	42 (1-7565)	8 (1-76)	53 (1-311)	506 (10-7565)	42 (4-2417)
Serum creatinine (μmol/l)	978 (98)	97 (56-1278)	83 (56-129)	90 (62-128)	131 (60-1278)	150 (59-1176)
GFR (ml· min⁻¹· 1.73 m⁻²)	957 (96)	77 ± 1	95 ± 1	96 ± 2	63 ± 2	50 ± 2
Creatinine clearance (ml· s⁻¹· 1.73 m⁻²)	578 (58)	1.32 ± 0.02	1.62 ± 0.02	1.51 ± 0.04	1.02 ± 0.04	0.81 ± 0.07
Urinary sodium excretion (mmol/day)	671 (67)	154 ± 3	152 ± 4	165 ± 6	154 ± 4	135 ± 9
Urinary potassium excretion (mmol/day)	671 (67)	87 ± 1	89 ± 2	93 ± 3	81 ± 2	83 ± 5
Serum total cholesterol (mmol/l)	928 (93)	5.2 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	5.1 ± 0.1	5.6 ± 0.1
Serum HDL cholesterol (mmol/l)	899 (90)	1.5 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
Serum LDL cholesterol (mmol/l)	868 (87)	3.1 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	3.4 ± 0.1	3.3 ± 0.1
Serum triglycerides (mmol/l) male	491 (91)	1.5 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
female	408 (89)	1.2 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.6 ± 0.1

Data are means ± SEM, median (range) or n (%). For abbreviations, see page 7. [a]Data based on one timed 24-hour urinary collection measured centrally. Patients with previously abnormal UAER had responded to antihypertensive treatment and showed regression of UAER at the investigation.

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Their clinical characteristics are shown in **Table 8**. Subjects from Helsinki University Central Hospital, Department of Medicine, Division of Nephrology (n=217), were recruited between November, 1994 and June, 1999, and all others (n=779) between March, 1998 and June, 1999. Clinical descriptions of the subjects in each referral center are in **Table 9**.

Table 9. Clinical characteristics by referral center

Hospital	n	Males	NO	MI	PR	ES	CVD	Age	DUR	HbA$_{1c}$	GFR
All	996	54	32	17	33	19	11	41 ± 10	28 ± 8	8.5 ± 1	77 ± 33
Helsinki University CH	217	59	23	8	35	35	15	43 ± 9	30 ± 8	8.6 ± 1	65 ± 39
Satakunta CH	109	48	35	15	37	14	10	40 ± 11	27 ± 8	8.3 ± 1	73 ± 30
Seinäjoki CH	93	56	52	18	25	5	8	42 ± 10	28 ± 9	8.6 ± 1	87 ± 27
Savonlinna CH	72	54	50	17	25	8	8	42 ± 9	27 ± 8	8.0 ± 1	85 ± 29
Päijat-Häme CH	56	54	29	13	41	18	7	38 ± 10	26 ± 8	7.9 ± 1	79 ± 32
Central Ostrobothnian CH	54	54	52	31	9	7	9	40 ± 11	28 ± 8	8.1 ± 1	90 ± 22
CH of Lansi-Pohja	54	50	46	24	26	4	9	40 ± 10	26 ± 7	9.4 ± 2	85 ± 28
CH of Kanta-Hame	50	54	8	24	50	18	14	44 ± 9	29 ± 8	8.6 ± 1	66 ± 31
Kainuu CH	47	62	38	26	30	6	9	38 ± 9	25 ± 9	8.9 ± 2	89 ± 28
North Karelian H	43	44	58	12	21	9	0	40 ± 11	27 ± 8	7.9 ± 1	84 ± 26
Vasa CH	41	66	0	24	59	17	12	39 ± 10	27 ± 8	8.9 ± 1	72 ± 35
Turku University CH	33	52	0	0	0	100	21	45 ± 8	33 ± 9	8.6 ± 2	53 ± 29
South Karelia CH	32	41	31	16	47	8	13	38 ± 11	27 ± 9	8.1 ± 1	80 ± 30
Tampere University H	25	40	0	12	68	20	20	38 ± 8	28 ± 7	9.3 ± 1	60 ± 20
CH of Kymenlaakso	22	82	36	41	23	0	0	32 ± 8	22 ± 8	8.4 ± 1	104 ± 22
Central Finland CH	19	32	53	11	21	16	5	34 ± 10	24 ± 9	8.5 ± 1	88 ± 32
Mikkeli CH	18	56	0	33	61	6	11	38 ± 11	26 ± 9	9.0 ± 2	79 ± 33
Lapland CH	6	67	67	33	0	0	0	44 ± 6	31 ± 6	8.5 ± 1	94 ± 20
City of Turku HC	4	0	50	0	50	0	25	51 ± 7	35 ± 9	8.4 ± 1	56 ± 23
CH of Åland Islands	1	100	0	0	1	0	0	58 ± 0	46 ± 0	7.5 ± 1	37 ± 0

Male column and classes by UAER are in %, all other columns are values ± SD. NO=normoalbuminuria, MI=microalbuminuria, PR=proteinuria, ES=end-stage renal disease, DUR=duration of diabetes, GFR=glomerular filtration rate according to Cockcroft-Gault formula, CH=central hospital, H=hospital, HC=health center.

4.2. Ethical aspects and informed consent

The study protocol followed the ethical principles expressed in the Declaration of Helsinki. The Ethics Committee of the Helsinki University Central Hospital endorsed the protocol in 1997. In addition, the local ethics committees of all participating hospital districts approved the study. All subjects received written information about the study prior to their participation and gave their written consent.

4.3. Study design

All studies had a cross-sectional, case-control study design. All the 996 subjects took part in each study, and were divided into four groups according to their UAER. Genotype and allele frequencies as well as haplotypes were compared between patients in the four groups, or between clinical variables biologically appropriate for each gene. Additionally, males and females were analyzed separately in all studies. The Hardy-Weinberg distribution was assessed for each polymorphism and each patient group.

4.3.1. Study I

In Study I, two common functional polymorphisms in the promoter region (–429 T/C and –374 T/A) and one in exon 3 (G82S) of the *RAGE* gene on chromosome 6p21.3 were genotyped. The genotypes, allele frequencies, and genotype combinations were analyzed with respect to nephropathy, renal function, retinopathy, CVD, AHT, serum lipids, diabetes duration, and glycemic control. Separate analyses were performed for the genotypes based on glycemic control between patients with normal UAER and those with proteinuria. Any variables showing a positive association were tested separately in multiple regression analyses.

4.3.2. Study II

The focus in Study II was on three polymorphisms in the nephrin gene (*NPHS1*) on chromosome 19q13.1. Thus, the E117K in exon3, R408Q in exon 10, and N1077S in exon 27 were genotyped for an association study in which the genotypes, allele frequencies, and genotype combinations were compared along with proteinuria and duration, to the diagnosis of microalbuminuria, proteinuria, or ESRD. Furthermore, the study tested whether any differences emerged in serum creatinine values, UAER levels, or serum creatinine clearance between the genotypes. Any possible association between any genotype combination and these variables was also assessed. In addition, three suggested polymorphisms (T295I, E447K, and R1140C in exons 8, 11, and 27, respectively) were genotyped and tested in 82 patients in order to elucidate whether they were true polymorphisms or were rare variants present only in the relatives of patients with congenital nephrosis.

4.3.3. Study III

Study III concerned genotyping of the functional Leu7Pro polymorphism of prepraneuropeptide Y. Analyses—performed in all patients and separately in a constellation, excluding those with ESRD—determined whether geno-

types or allele frequencies were associated with serum lipids, glycemic control, arterial BP, AHT or CVD. All variables with a positive association were entered into a multiple regression analysis to test whether they were independently associated.

4.3.4. Study IV

Study IV focused on the *DRD3* gene on chromosome 3q13.3. In this gene, two polymorphisms located in exon 1 (Ser9Gly and Ala17Ala) and one in the promoter region (–707 G/C) were genotyped. Analyses showed whether any association existed between the geno-

types, allele frequencies or haplotypes and BP levels, AHT, CVD and variables involved with renal function.

4.3.5. Study V

Study V involved genotyping of one functional polymorphism in intron 1 (G1675A), and one in intron 2 (A1818T) of the *AT2* gene. Because of its X-chromosomal location, all analyses were performed separately between males and females. The genotypes, allele frequencies, and haplotypes were compared with BP levels, glycemic control, renal function, and prevalence of CVD, AHT, diabetic nephropathy, and retinopathy.

5. METHODS

5.1. Phenotype assessments

Attending physicians and nurses caring for the patients collected clinical data from all subjects recruited for the FinnDiane study. All physicians were diabetologists or nephrologists and the nurses trained in diabetology or nephrology. All data—collected during a regular visit to the hospital—included a thorough medical history, BP, height, weight, and waist and hip measurement, as well as one timed 24-hour urine collection and drawing of blood samples. In addition, all patients completed a questionnaire concerning their medical history.

5.1.1. Medical history

The extensive information collected from these subjects concerned diabetes and late complications, hypertension, renal function, regular medication, previous laboratory values, chronic diseases other than diabetes, and socio-economic factors, as well as the medical history of parents and siblings. Questionnaires were designed particularly for the FinnDiane study.

A patient was classified as having type 1 diabetes if the diagnosis was made prior to the 40th birthday and if insulin treatment was initiated within one year,

and if any oral antidiabetic medication ceased within one year of diagnosis. Based on a complete list of regular medications, patients with treatment potentially affecting kidney function were excluded. Smoking was defined as present smoking of at least one daily cigarette, cigar, or pipe during the year prior to participation. The patient was defined as an ex-smoker if more than one year had passed since cessation of smoking.

All local laboratory values available from the last 10-year period and relevant to diabetes and late diabetic complications went to the FinnDiane coordinating center in Helsinki. Variables requested were: HbA_{1c}, serum total cholesterol, serum triglycerides, serum high-density lipoprotein (HDL) cholesterol, and serum creatinine. LDL cholesterol was calculated by the Friedewald formula (267). Similarly, all available local urinary albumin measurements provided were used for the classification of nephropathy.

5.1.2. Classification of nephropathy

The physician in charge of each patient reported whether the patient had previously been diagnosed with microalbuminuria, overt proteinuria, or ESRD. However, the final classification into 1)

Normal UAER (normoalbuminuria), 2) Microalbuminuria, 3) Proteinuria (overt nephropathy), or 4) ESRD was performed centrally and assessed according to the UAER in at least two of three past consecutive overnight or 24-hour urine collections. All patients with a normal UAER (< 20 µg/min or < 30 mg/24 hours, n = 317) were required to have had a diabetes duration more than 15 years. A total of 164 patients had microalbuminuria (AER 20-200 µg/min or 30-300 mg/24 hours), 325 had proteinuria (UAER > 200 µg/min or > 300 mg/24 hours), and 184 ESRD. All ESRD patients were further classified as on dialysis (n=44) or with a kidney transplant (n=140). If abnormal UAER was diagnosed during pregnancy only, these values were not taken into consideration in the classification procedure.

5.1.3. Retinopathy

Retinopathy was recorded either as any retinopathy or proliferative retinopathy. The patient was considered to have proliferative retinopathy if he or she had undergone retinal photocoagulation.

5.1.4. Blood pressure and hypertension

Systolic (SBP) and diastolic (DBP) clinically tested office blood pressure, registered as Korotkoff I and V sounds, was measured in each center with a calibrated mercury sphygmomanometer. Two measurements were performed, each after at least 10 minutes of rest. The mean value served for statistical analysis. Pulse pressure was calculated as SBP minus DPB. Hypertension was defined as regular use of at least one antihypertensive agent such as an angiotensin-converting enzyme (ACE) inhibitor, angiotensin II type 1 receptor inhibitor, diuretic, calcium-channel inhibitor, or beta-blocker.

5.1.5. Cardiovascular disease

Information about CVD came from medical records and included verified CHD, AMI, or stroke. The patients were considered to have CHD if they had suffered an AMI reported by their physician, undergone a coronary by-pass or percutaneous transluminal coronary angioplasty (PTCA), or if they were permanently using short- or long-acting nitroglycerin or both. The patients qualified for AMI if they had signs of a previous myocardial infarction in the electrocardiogram assessed by the physician. All classified as having PVD were required either to have had bypass surgery performed on either leg or to have had at least one toe or leg amputated.

5.1.6. Anthropometric measurements and insulin sensitivity

Body weight was registered to the closest 0.1 kg and height to the closest 1 cm, in light clothing without shoes. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Waist circumference was measured midway between the iliac crest at the midaxillary line and the lowest rib; hip circumference was at the widest part of the gluteal region. Waist-to-hip ratio (WHR) was calculated as waist/hip (cm/cm) and served as an indicator of central obesity. Estimated glucose disposal rate (eGDR), a surrogate for insulin sensitivity, was calculated according to the formula (mg x kg^{-1} x min^{-1}), as suggested by Williams *et al.* (268).

5.2. Sample collection and laboratory assays

Of 85 ml of blood drawn by venopuncture, 60 ml was drawn into six EDTA tubes for genetic analyses, as described in section 5.3; 20 ml was drawn into two serum tubes and 5 ml into a tube containing aprotinin. Patients were also asked for a 24-hour urine sample. Blood and urine samples were immediately deep frozen and stored at -20° C. Urinary albumin concentration was measured centrally with RIA (Pharmacia, Uppsala, Sweden). Serum and urine creatinine

concentration levels for calculation of creatinine clearance were measured by the modified Jaffé reaction with a Hitachi 911 E Automatic Analyser (Boehringer Mannheim, Mannheim, Germany) and serum C-peptide concentrations with a Human C-peptide RIA kit (Linco Research Inc, St Charles, MO, USA), at the central laboratory. Glomerular filtration rate (GFR) was calculated by the Cockcroft-Gault formula (269).

HbA$_{1c}$, serum cholesterol, and serum triglycerides were measured by routine methods at the local laboratory of each referral center. Normal range for HbA$_{1c}$ was 4.0 to 6.0% in 75% of the local laboratories, and in all centers the upper normal value was below 7.0%. For triglycerides, the normal range was 0.4 to 1.7 mmol/l in 55% of the centers, and in all centers the upper normal range was below 2.0 mmol/l in 90% and below 3.0 mmol/l. For total serum cholesterol, the normal range was < 5.0 mmol/l in 40% of the centers, and the upper normal range was below 7.0 mmol/l in 95%. For serum HDL cholesterol these were, respectively, > 1.0 mmol/l in 40% and > 0.8 mmol/l in 100% of the centers. All local laboratories had participated in a national quality assessment program.

Plasma NPY was measured centrally with a commercial radioimmunoassay (RIA) kit EURIA-NPY (Euro-Diagnostica Inc., Malmö, Sweden). From the 24-hour urine collection, urinary sodium (Na$^+$) and potassium (K$^+$)

were measured centrally by a standard-ized ion selective electrode technique (normal ranges, 130-240 mmol/l and 60-90 mmol/l, respectively).

5.3. Genotyping

Deoxyribonucleic acid (DNA) was ex-tracted from whole blood according to standard protocols with slight modifica-tions (270). In all studies, genotyping was performed by the minisequencing method, in which one of the primers in each polymerase chain reaction (PCR) was biotinylated (271,272), except for the Leu7Pro polymorphism in Study III where a *Bsi*EI restriction enzyme was used (133). The PCR primers were or-dered form the Genethon Database (273) except for Study III (133). In all studies, the PCR protocol was run in 96-well microtitration plates by use of a MJ Research programmable thermal cycler (MJ Research, Watertown, MA, USA).

Study I. For amplification of the region containing the –429 T/C and –374 T/A polymorphisms in the *RAGE* gene, primers were as described (274), but with an added biotinylated F5' end. Minisequencing of the –429 T/C and –374 T/A was determined with reverse minisequencing primers 5'-AGGAGAGAAACCTGTTTGGA-3' and 5'-CTGTTGTCTGCAAGGGTGCA-3', respectively. The G82S primers have

been described (275), with the forward minisequencing primer 5'-TGGCTCGTGTCCTTCCCAACG-3'.

Study II. The PCR primers for all polymorphisms in the nephrin gene were identical to those used by Lenkkeri *et al.* (261). For the E117K polymor-phism located in exon 3 was used a reverse minisequencing primer: 5'-CACGAGCTCGGGCCCCATCT-3', but for the R408Q and N1077S polymor-phisms located in exon 10 and 24, re-spectively, a forward minisequencing primer 5'-CTGACATTCCTGGCGCGG-3' and 5'-GGGCTTCTGCTCCTCTCC-3'. Genotyping of the E117K polymorphism failed in 35 patients (3% of the total study population) because of spurious results in the minisequencing reaction, whereas for the R408Q and N1077S polymorphisms, PCR failure occurred only in one and in five patients. Fur-thermore, three suggested polymor-phisms: the T295I in exon 8, E447K in exon 11, and R1140C in exon 27, were genotyped according to previously de-signed PCR primers (276,277) using forward minisequencing primers of 5'-AGCGTGGGGCACAGAGCACA-3', 5'-CCGCCCAGAAACTGTGGATT-3', and 5'-CAGAGGCAGAGCCGTATTAC-3', respectively.

Study III. The Prepro-NPY Leu7Pro genotypes were determined according to a previously described method (133). Due to failure of the PCR reaction, one

patient remained ungenotyped.

Study IV. The Ser9Gly and the Ala17Ala sites located on exon 1 of the *DRD3* gene were included in the same PCR reaction using the forward primer 5'-TTTCTGTCTCCTCACAGGAA-3' and reverse primer 5'-CAGCAGGCCATTGCCGAA-3'. Large sections with no previously known polymorphisms in the *DRD3* coding region, promoter, and 3' end were systematically sequenced by another group at the same institution (unpublished data) using standard sequencing procedures in 12 healthy control patients. Only one previously unknown A to G substitution in codon 17 (Ala17Ala) at base position 278 bp (GENEBANK accession U25441) was discovered. The forward minisequencing primers in the Ser9Gly and Ala17Ala variants were 5'-CATCTCTGAGTCAGCTGAGT-3' and 5'-CTGAACTACACCTGTGGGGC-3'. The PCR primers in the −707 G/C polymorphism have been described (278), in which the 5'-TGGGAAGAATCTGGAGCTCA-3' minisequencing primer is used for genotyping of this polymorphism.

Study V. The PCR primers of the G1675A and the A1818T polymorphisms of the *AT2* gene have been described elsewhere (279). For these polymorphisms, the minisequencing primers were 5'-CTGTATTTTGCAAAACTCCT-3' and 5'-TTATGTTAATTTGTTAGGTC-3'. Genotyping of the G1675A polymor-

phism failed in two patients due to PCR failure.

5.4. Statistical methods

5.4.1. General

Descriptive data, unless otherwise stated, are expressed as standard error of mean (± SEM), standard deviation (± SD), or median value (range). Categorical variables were compared by the chi-squared test. Normally distributed continuous variables were subjected to Student's t-test, while non-normally distributed variables were logarithmically transformed or assessed by the Mann-Whitney U-test. Differences between multiple groups were tested with analysis of variance (ANOVA), but with the Kruskal-Wallis test if the variables were not normally distributed. To evaluate any independent association between variables for any dependent categorical variable, a backward stepwise multiple logistic regression model was used with a means substitution of absent data. Between variables for any dependent continuous variable, a backward stepwise multiple linear regression model was used. All statistical analyses were also performed separately for each gender. Analyses were carried out with the STATISTICA 4.1 statistical package (Tulsa, OK, USA).

5.4.2. Linkage disequilibrium and haplotypes

In Studies I and II, the linkage disequilibrium (LD) analyses were performed with the LinkDos and Genepop packages (version 1.2) (280) and the distribution of genotype combinations by the MENDEL program (281). In Study IV, LD analyses between the markers were performed by use of the 2 by 2 program, version 1.50 (282), and haplotypes were determined using the PHASE version 2.0.1 (283). In Study V, LD was analyzed with the Linkage Disequilibrium Analyzer 1.0 (284), and haplotypes were determined by PHASE version 2.0.2 (283). The haplotypes in Studies IV and V were imported into a STATISTICA 4.1 file and compared with all variables of interest. Males and females were always analyzed separately in Study V.

6. RESULTS

6.1. *RAGE* polymorphisms in relation to proteinuria and cardiovascular disease (I)

The *RAGE* –374 T/A and G82S polymorphisms were in Hardy-Weinberg equilibrium for all four groups divided according to their UAER. However, the –429 T/C polymorphism deviated significantly from equilibrium ($c^2 = 20.8$, p < 0.0001). The frequencies of the homozygous mutant genotype for the –429 T/C, –374 T/A, and G82S polymorphisms were 2, 13, and 1%, respectively.

No differences in genotypes or allele frequencies appeared between the four groups. AA genotype frequency of the –374 T/A polymorphism was 15% in patients with normal UAER compared to 12% in those with proteinuria (p = NS). In patients with poor glycemic control ($HbA_{1c} > 9.5$), however, the AA genotype of the –374 T/A polymorphism was significantly more common in subjects with a normal UAER (30%, n = 27) than in those with proteinuria (10%, n = 91) (**Figure 3**).

In a similar analysis, HbA_{1c} levels were unaffected by genotype frequencies of the –429 T/C and G82S polymorphisms (data not shown).
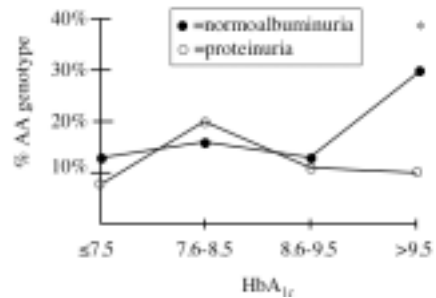
Figure 3. Patients (%) with the homozygous minor genotype (AA) of the *RAGE* –374 T/A polymorphism. All patients with normal UAER and proteinuria analyzed with their HbA_{1c} by interval. *P = 0.01.

Table 10. Genotype distribution and macrovascular complications by –374 T/A polymorphism in all patients

Variable	AA	TT+TA	P
CVD+	6 (6)	100 (94)	
CVD-	124 (14)	757 (86)	0.01
CHD+	5 (6)	80 (94)	
CHD-	120 (14)	752 (86)	<0.05
AMI+	1 (2)	52 (98)	
AMI-	128 (14)	804 (86)	0.01
PVD+	1 (2)	40 (98)	
PVD -	129 (14)	815 (86)	<0.05

All data given as n (%). P-value by two-tailed Fisher's exact test. For abbreviations, see page 7.

Among the 106 patients with manifest CVD, it was less common in those with the AA genotype than with the TT and TA genotypes of the –374 T/A polymorphism. In addition, patients with the AA genotype had less CHD, fewer AMIs, and less PVD (**Table 10**).

Furthermore, the AA genotype of the –374 T/A polymorphism was independently associated with CVD (p = 0.009, R = 0.40, beta = -0.983) in the multiple logistic backward regression analysis. The model also included gender, age, HbA_{1c}, serum triglycerides, waist-to-hip ratio, current smoking, AHT, and parental CVD. In addition to the *RAGE* –374 T/A polymorphism, AHT (p < 0.001, beta = 1.819), HbA_{1c} (p = 0.008, beta = 0.137), and age (p < 0.001, beta = 0.093) were associated independently with CVD.

6.2. Nephrin gene polymorphisms and diabetic nephropathy (II)

The three polymorphisms of the nephrin gene were in Hardy-Weinberg equilibrium for all other groups except for the N1077S polymorphism in subjects with microalbuminuria, where a slight deviation was observed (χ^2 = 4.34, p < 0.05). The minor allele frequencies in the E117K, R408Q, and N1077S polymorphisms, located in exons 3, 10, and 24, were 34, 8, and 12% in the entire cohort. No differences in genotype- or in allele frequencies were evident between any of the four groups when they all were analyzed together, or when the genotype- or the allele frequencies were analyzed separately for each group (**Table 11**). Power calculations indicated that the cohorts were sufficiently large to yield

Table 11. Distribution of genotypes and rare allele frequencies of nephrin polymorphisms

Polymorphism	NORMO	MICRO	PROT	ESRD	P
E117K					NS
EE	146 (46)	73 (44)	140 (44)	65 (40)	
EK	132 (41)	75 (46)	134 (43)	68 (42)	
KK	40 (13)	17 (10)	41 (13)	30 (18)	
K	0.33	0.33	0.34	0.39	NS
R408Q					NS
RR	273 (85)	140 (84)	267 (82)	156 (85)	
RQ	45 (14)	26 (16)	58 (18)	24 (13)	
QQ	3 (1)	0 (0)	0 (0)	3 (2)	
Q	0.08	0.08	0.09	0.08	NS
N1077S					NS
NN	242 (76)	128 (77)	253 (78)	145 (78)	
NS	74 (23)	32 (19)	67 (21)	38 (21)	
SS	3 (1)	6 (4)	3 (1)	1 (1)	
S	0.13	0.13	0.11	0.11	NS

Genotypes as n (%). For abbreviations, see page 7.

80% power to detect a 9% deviation in rare genotype frequency at p < 0.05, and had 99.5% power to detect a 14% deviation in genotype difference. All three polymorphisms were in LD with each other (p < 0.0001). With respect to the T295I, E447K, and R1140C polymorphisms in exon 8, 11, and 27, none among the 82 first patients genotyped possessed the minor allele, and thus no further genotyping was performed.

Patients with the wild genotype and patients with one or two rare alleles of each polymorphism showed no difference in length of time from onset of diabetes to diagnosis of nephropathy or onset of ESRD, or in any of the measured renal variables (**Table 12**). No genotype combination was associated with ESRD, proteinuria, or AHT, but the genotypes E117E, R408Q, and N1077N were more prevalent in ESRD individuals with CHD cases than in the other genotypes (p = 0.04). This positive association disappeared, however, after the correction for multiple testing.

Table 12. Clinical by nephrin genotypes

Polymorphism	Wild genotype	Minor genotypes	P-value
	GFR (ml·min^{-1}·1.73 m^{-2})[a]		
E117K	83 ± 28	81 ± 30	NS
R408Q	83 ± 29	79 ± 30	NS
N1077S	82 ± 30	83 ± 30	NS
	Urinary sodium excretion (mmol/day)[a]		
E117K	154 ± 65	153 ± 70	NS[b]
R408Q	156 ± 69	153 ± 62	NS[b]
N1077S	156 ± 69	155 ± 65	NS[b]
	Urinary potassium excretion (mmol/day)[a]		
E117K	85 ± 33	88 ± 33	NS[b]
R408Q	87 ± 69	87 ± 62	NS[b]
N1077S	88 ± 33	85 ± 33	NS[b]
	UAER (mg/24 h)[c]		
E117K	665 ± 1188	832 ± 1258	NS
R408Q	725 ± 1159	916 ± 1530	NS
N1077S	882 ± 1225	655 ± 1221	NS
	Time from onset of DM to nephropathy (years)		
E117K	19 ± 7	19 ± 8	NS
R408Q	19 ± 7	19 ± 8	NS
N1077S	19 ± 7	20 ± 7	NS
	Time from onset of DM to ESRD (years)		
E117K	26 ± 8	26 ± 7	NS
R408Q	26 ± 8	27 ± 7	NS
N1077S	26 ± 7	28 ± 9	NS

Data are ± SD. DM=type 1 diabetes. [a]Patients with ESRD excluded. [b]P = NS when only patients without permanent medication except for insulin (n=182) were included. [c]Only patients with microalbuminuria and proteinuria included.

6.3. *NPY* Leu7Pro polymorphism and late complications (III)

Genotype frequencies for all four groups were in Hardy-Weinberg equilibrium. The frequency of the Pro7 substitution was 11% for patients with normal UAER, 10% for those with microalbuminuria, 16% for those with proteinuria, and 13% for patients with ESRD ($\chi^2 = 5.48$, df = 3, p = 0.14). The Pro7 substitution was more common in patients with overt nephropathy than in patients with normal UAER ($\chi^2 = 4.00$, df = 1, p < 0.05).

HbA$_{1c}$, triglycerides, BMI, and CVD prevalence were significantly higher in subjects with the Pro7 substitution than in those with the Leu7/Leu genotype (**Table 13**). Since the majority of patients with ESRD had received a renal transplant and thus had a normal UAER at the time of investigation, and since this group also has a potential survival bias, data presented do not include patients with ESRD. In addition, total cholesterol was higher and eGDR lower in patients with the Pro7 substitution. Therefore, it was not unexpected that insulin dose was also higher in females with the Pro7 substitution. No differences between genotypes appeared in SBP or DBP, PP, or prevalence of AHT, AMI, or stroke.

Plasma NPY was 111.5 ± 3.0 nmol/l in those with the wild type genotype and 110.3 ± 4.4 nmol/l in those with the Pro7 substitution (p = NS). No differences were detectable when males and females were analyzed separately, nor were there any differences when subjects with proteinuria and normal UAER were analyzed separately nor when the patients were divided into quintiles according to their plasma NPY. The plasma NPY was 96.7 ± 2.7 nmol/l in those with normal UAER in comparison to 121.3 ± 3.5 nmol/l in those with proteinuria (p < 0.001). Positive correlations appeared between plasma NPY and HbA$_{1c}$ (r = 0.24, p < 0.001), total cholesterol (r = 0.40, p < 0.001), LDL cholesterol (r = 0.23, p < 0.001), and serum creatinine (r = 0.51, p < 0.001). The Leu7Pro genotype was also independently associated with proteinuria, CHD and HbA$_{1c}$ in multiple regression analyses (**Table 14**).

Table 13. Clinical variables by Leu7Pro polymorphism of the *NPY*, with ESRD patients excluded (n=811)

Variable	All			Male			Female		
	Leu7Leu	Pro7/-	P	Leu7Leu	Pro7/-	P	Leu7Leu	Pro7/-	P
n (%)	706 (87)	105 (13)	-	371 (87)	57 (13)	-	335 (87)	48 (13)	-
Age (years)	40.0 ± 0.4	40.0 ± 1.2	NS	41.5 ± 0.5	41.8 ± 1.3	NS	39.7 ± 0.5	38.7 ± 1.5	NS
Diabetes duration (years)	26.9 ± 0.3	27.0 ± 0.9	NS	26.7 ± 0.4	28.0 ± 1.3	NS	27.0 ± 0.4	25.8 ± 1.2	NS
BMI (kg/m^2)	25.3 ± 0.1	26.1 ± 0.4	<0.05	25.6 ± 0.2	25.9 ± 0.6	NS	25.0 ± 0.2	26.3 ± 0.6	<0.05
WHR	0.872 ± 0.004	0.882 ± 0.009	NS	0.921 ± 0.004	0.927 ± 0.009	NS	0.817 ± 0.004	0.826 ± 0.011	NS
CVD (%)	46 (7)	12 (12)	<0.05	31 (8)	6 (11)	NS	15 (4)	6 (13)	<0.05
CHD (%)	37 (5)	11 (11)	NS	22 (6)	6 (11)	NS	15 (5)	5 (11)	NS[a]
GFR (ml·min^{-1}·1.73 m^{-2})[b]	82.2 ± 1.1	80.3 ± 3.2	NS	88.0 ± 1.7	82.9 ± 4.9	NS	75.8 ± 1.3	77.0 ± 3.8	NS
HbA$_{1c}$ (%)[b]	8.5 ± 0.1	9.0 ± 0.1	<0.001	8.6 ± 0.1	9.0 ± 0.2	NS	8.3 ± 0.1	9.0 ± 0.2	0.001
eGDR (mg · kg^{-1} · min^{-1})	6.13 ± 0.10	5.61 ± 0.26	NS	5.11 ± 0.13	4.92 ± 0.31	NS	7.27 ± 0.14	6.43 ± 0.40	<0.05
Insulin dose (IU · kg^{-1})[c]	0.70 ± 0.01	0.73 ± 0.02	NS	0.72 ± 0.01	0.70 ± 0.03	NS	0.68 ± 0.01	0.77 ± 0.03	<0.05
Total cholesterol (mmol/l)	5.10 ± 0.04	5.38 ± 0.13	<0.05	5.10 ± 0.04	5.44 ± 0.17	NS	5.12 ± 0.06	5.29 ± 0.15	NS
HDL cholesterol (mmol/l)	1.52 ± 0.02	1.50 ± 0.06	NS	1.37 ± 0.02	1.33 ± 0.05	NS	1.71 ± 0.03	1.70 ± 0.10	NS
LDL cholesterol (mmol/l)	3.04 ± 0.04	3.03 ± 0.10	NS	3.12 ± 0.05	3.18 ± 0.12	NS	2.95 ± 0.05	2.86 ± 0.16	NS
Triglycerides (mmol/l)[d]	1.27 ± 0.03	1.72 ± 0.13	<0.0001	1.42 ± 0.05	1.82 ± 0.21	<0.05	1.10 ± 0.03	1.60 ± 0.13	<0.0001

Data are mean ± SEM, or n (%). For abbreviations, see page 7. [a]Fisher's exact two-tailed test. [b]$p < 0.001$, $p = $ NS, and $p < 0.005$ in all, in males, and in females, respectively, when adjusted for BMI. [c]$p = $ NS in all, in males, and in females, respectively, when adjusted for HbA$_{1c}$. [d]$p = 0.001$, $p = $ NS, and $p < 0.005$ in all, in males, and in females, respectively, when adjusted for HbA$_{1c}$.

Table 14. Multiple backward stepwise regression analyses

A. Linear regression with HbA$_{1c}$ as dependent variable. Patients with ESRD excluded. Complete data available from 679 patients. Other variables included in the first model: sex, serum creatinine, triglycerides, SBP, and lipid-lowering medication. Adjusted R^2 = 0.14.

Variable	Coefficient	SEM	P
Age	-0.023	0.005	<0.001
Triglycerides	1.205	0.260	<0.001
LDL cholesterol	0.290	0.060	<0.001
WHR	1.492	0.617	0.016
NPY Leu7Pro	0.555	0.156	<0.001

B. Logistic regression with proteinuria vs. normal UAER as dependent variable. Complete data available from 679 patients. Other variables included in first model: sex, BMI, and current smoking. R^2 = 0.69.

Variable	Coefficient	SEM	P
Diabetes duration	-0.031	0.015	0.036
HbA$_{1c}$	0.207	0.090	0.022
Triglycerides	2.942	0.613	<0.001
LDL cholesterol	0.543	0.168	0.001
AHT	5.287	0.598	<0.001
NPY Leu7Pro	1.250	0.457	0.006

C. Logistic regression with CHD as dependent variable in all patients. Complete data available from 957 patients. Other variables included in first model: sex, LDL cholesterol, WHR, and current smoking. R^2 = 0.14.

Variable	Coefficient	SEM	P
Age	0.092	0.010	<0.001
HbA$_{1c}$	0.160	0.055	<0.005
AHT	1.645	0.365	<0.001
NPY Leu7Pro	0.634	0.229	0.006

For abbreviation, see page 7.

6.4. *DRD3* gene polymorphisms, blood pressure, and diabetic nephropathy (IV)

All *DRD3* polymorphisms studied were in Hardy-Weinberg equilibrium for all four patient groups. Frequencies for the minor alleles were 18, 30, and 10% for the –707 G/C, Ser9Gly, and Ala17Ala genotypes, respectively. Power calculations showed that cohorts were sufficiently large to yield 80% power to detect an 11% deviation in rare genotype frequency at p < 0.05, and had 99.5%

power with one marker to detect a 13% deviation between the four groups as shown by power calculations. The –707 G/C and Ala17Ala were in tight LD with the Ser9Gly (D = 0.13, D' = 1.00 and D = 0.07, D' = 0.98, respectively, p < 0.0001).

No differences in genotypes or in allele frequencies were evident among the four groups (**Table 15**).

Table 15. Distribution by genotypes, minor allele frequencies, and haplotypes of the *DRD3* polymorphisms according to UAER

	NORMO	MICRO	PROT	ESRD	P
Polymorphism					
-707 G/C: GG	210 (65)	113 (68)	233 (72)	113 (61)	
GC	102 (32)	46 (28)	82 (25)	62 (34)	
CC	9 (3)	7 (4)	10 (3)	9 (5)	NS
C	0.19	0.18	0.16	0.22	NS
Ser9Gly: AA	153 (48)	81 (49)	174 (54)	80 (43)	
AG	140 (44)	70 (42)	123 (38)	80 (44)	
GG	28 (9)	15 (9)	28 (9)	24 (13)	NS
G	0.31	0.30	0.28	0.35	NS
Ala17Ala: AA	263 (82)	134 (81)	263 (81)	145 (79)	
AG	53 (17)	29 (17)	59 (18)	38 (21)	
GG	5 (2)	3 (2)	3 (1)	1 (1)	NS
G	0.10	0.11	0.10	0.11	NS
Haplotypes					
G-Ser-A	446 (70)	232 (70.)	470 (72)	238 (65)	NS
C-Gly-A	120 (19)	60 (18)	102 (16)	80 (22)	NS
G-Gly-G	63 (10)	35 (11)	64 (10)	38 (10)	NS
G-Gly-A	13 (2)	5 (2)	13 (2)	10 (3)	NS
G-Ser-G	0	0	1 (0)	2 (1)	NS

Genotype and haplotype data are n (%). For abbreviations, see page 7. Haplotype order: -707 G/C, Ser9Gly and Ala17Ala (A/G).

We observed five haplotype combinations but no differences between the groups (**Table 15**). In addition to the variables in **Table 16**, no differences appeared in the prevalence of AHT, CVD, or CHD in any of the polymorphisms when comparing patients with the wild genotype to patients with one or two minor alleles. Neither were there any associations between the genotypes and endpoints like initiation of AHT, onset of diabetic nephropathy, or ESRD.

Table 16. Clinical variables by *DRD3* genotype

Polymorphism	Wild genotype	Minor genotypes	P
	SBP (mmHg)		
-707 G/C	140 ± 20	141 ± 19	NS
Ser9Gly	140 ± 20	141 ± 20	NS
Ala17Ala	141 ± 17	141 ± 19	NS
	DBP (mmHg)		
-707 G/C	82 ± 10	82 ± 10	NS
Ser9Gly	82 ± 10	82 ± 10	NS
Ala17Ala	82 ± 10	82 ± 11	NS
	PP (mmHg)		
-707 G/C	59 ± 18	59 ± 17	NS
Ser9Gly	58 ± 18	59 ± 17	NS
Ala17Ala	59 ± 17	59 ± 19	NS
	GFR (ml· min^{-1}· 1.73 m^{-2})[a]		
-707 G/C	77 ± 32	76 ± 32	NS
Ser9Gly	77 ± 31	76 ± 33	NS
Ala17Ala	76 ± 31	77 ± 35	NS
	Urinary sodium excretion (mmol/day)[a,b]		
-707 G/C	153 ± 68	158 ± 66	NS
Ser9Gly	151 ± 68	157 ± 66	NS
Ala17Ala	155 ± 68	153 ± 60	NS
	Urinary potassium excretion (mmol/day)[a,b]		
-707 G/C	87 ± 35	85 ± 29	NS
Ser9Gly	83 ± 34	89 ± 32	0.05
Ala17Ala	85 ± 33	93 ± 32	0.02

Data are ± SD. For abbreviations, see page 7. [a]Patients with ESRD excluded. [b]Only patients with no permanent medication except for insulin (n=182) were analyzed.

6.5. *AT2* gene polymorphisms, diabetic nephropathy, and blood pressure (V)

Both the functional G1675A SNP in intron 1 and the probably non-functional A1818T in intron 2 of the *AT2* gene were in Hardy-Weinberg equilibrium for females but could not be assessed in males due to their X-chromosomal location. The minor allele frequencies were 0.45 and 0.28 in females and in males 0.46 and 0.27 for the G1675A and A1818T polymorphism, respectively. In LD analyses, D´ = 0.98 and r^2 = 0.31 in males, and D´ = 0.97 and r^2 = 0.30 in females. The haplotypes were distributed as GA (n = 250, 27%), GT (n = 251, 27%), AA (n = 412, 45%), and AT (n = 3, 0%) in 458 females. In 536 males they were GA (n = 146, 27%), GT (n = 142, 26%), AA (n = 247, 46%), and AT (n = 1, 0%) for the G1675A and A1818T polymorphism, respectively.

The G allele of the G1675A and the T allele of the A1818T and GT haplotypes were more common in male patients with normal renal function (GFR > 90 ml/min/1.73m^2) than in those with

impaired renal function (**Table 17**). The G allele of G1675A and the T allele of A1818T were also associated with a higher absolute GFR value when excluding patients with ESRD, and a lower PP and higher eGDR (**Table 18**).

However, none of these associations appeared in females. In addition, no association existed between the *AT2* genotype and UAER in either males or females (data not shown).

Table 17. Renal function compared with genotype

	GFR > 90	60 GFR 90	GFR < 60	Pᵃ
G1675A males				
G	132 (61)	53 (46)	99 (51)	
A	86 (39)	62 (54)	94 (49)	0.027
A1818T males				
A	149 (68)	95 (83)	143 (74)	
T	71 (32)	20 (17)	50 (26)	0.013
Haplotypes males				
GT	71 (45)	19 (24)	50 (35)	
AA	86 (55)	61 (76)	94 (65)	0.004
G1675A females				
G	113 (54)	219 (54)	144 (55)	
A	97 (46)	185 (46)	120 (45)	NS
A1818T females				
A	155 (74)	295 (73)	186 (70)	
T	55 (26)	109 (27)	78 (30)	NS
Haplotypes females				
GT	53 (36)	97 (36)	93 (40)	
AA	94 (64)	169 (64)	141 (60)	NS

Data are n (%). All ESRD patients were classified as GFR < 60 ml· min^{-1}· 1.73 m^{-2}. ᵃdf=2

Since the G allele of the G1675A and the T allele of the A1818T appeared to be protective, the haplotypes were analyzed as AA against GT. By this approach, males with a GT haplotype showed higher GFR and eGDR and lower PP than did those with an AA haplotype (**Table 18**). This difference remained statistically significant after adjusting for age. Furthermore, the G1675A allele was independently associated with PP (r^2 = 0.16, coefficient = 3.64, SE = 1.38, p < 0.01) after adjusting for HbA1$_c$, LDL

cholesterol, BMI, and AHT. Again, no association was seen in females.

Despite an association among the haplotypes of the *AT2* gene and renal function and BP in males, there were no differences in the genotypes nor in the haplotypes of the *AT2* gene to parallel the prevalence of retinopathy or macrovascular disease. Furthermore, for either genotype or haplotypes, in either gender, no differences were evident in time to onset of diabetic nephropathy or ESRD.

Table 18. Clinical characteristics by *AT2* genotype and haplotype in males[a]

Variable	G1675A			A1818T			Haplotypes		
	G	A	P	A	T	P	GT	AA	P
n (%)	288 (53.7)	244 (46.3)	-	395 (73.4)	143 (26.6)	-	142 (36.5)	247 (63.5)	-
Age (years)	40.7 ± 9.7	42.7 ± 10.3	<0.05	42.0 ± 10.0	40.3 ± 9.9	NS	40.2 ± 9.8	42.6 ± 10.2	0.024
Diabetes duration (years)	27.6 ± 8.6	28.6 ± 8.7	NS	28.4 ± 8.8	26.9 ± 8.2	NS	26.8 ± 8.1	28.5 ± 8.7	NS
HbA$_{1c}$ (%)	8.6 ± 1.4	8.7 ± 1.4	NS	8.6 ± 1.4	8.6 ± 1.4	NS	8.6 ± 1.4	8.7 ± 1.4	NS
eGDR (mg · kg^{-1} · min^{-1})	5.12 ± 2.26	4.51 ± 2.20	<0.005[b]	4.70 ± 2.24	5.22 ± 2.25	<0.05[c]	5.20 ± 2.25	4.50 ± 2.20	<0.005[b]
SBP (mmHg)	141 ± 19	145 ± 20	<0.05[c]	143 ± 20	142 ± 17	NS	142 ± 17	145 ± 20	NS
DBP (mmHg)	84 ± 10	83 ± 10	NS	83 ± 10	85 ± 11	0.05[c]	85 ± 11	83 ± 10	NS
PP (mmHg)	57 ± 17	62 ± 18	<0.001[b†]	61 ± 18	57 ± 15	<0.05[c]	57 ± 15	62 ± 18	<0.005[b]
UAER (mg· 24 h)[d]	67 (2-7565)	136 (1-5589)	NS	84 ± (1-5589)	68 (4-7586)	NS	68 (4-7565)	132 (1-5589)	NS
GFR (ml· min^{-1}· 1.73 m^{-2})[d]	90.4 ± 32.4	83.4 ± 32.1	<0.05[e]	85.0 ± 32.0	93.7 ± 32.8	<0.05[e]	93.8 ± 32.9	83.4 ± 32.1	<0.01[f]
CHD (%)	22 (8)	34 (14)	<0.05[c]	48 (13)	9 (7)	<0.05[c]	9 (7)	34 (14)	<0.05[c]

Data as mean ± SD, median (range) or n (%). For abbreviations, see page 7. [a]Data are genotypes and allele frequencies simultaneously due to the X-chromosomal location. [b]P < 0.05 when adjusted for age. [c]P = NS NS with adjustment for age. [d]Patients with ESRD excluded. [e]P = NS (G1675A) and P = 0.005 (A1818T) with adjustment for duration of diabetes, use of ACE inhibitors, and AER.

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7. DISCUSSION

7.1. Study subjects and their classification

This study is part of the ongoing FinnDiane Study, a comprehensive multicenter, nationwide project with the aim to characterize 25% of all adult patients with type 1 diabetes in Finland. The patients were recruited from the first 20 referral centers that joined the FinnDiane study between 1994 and 1999, and these centers covered all five university hospital districts in Finland. All centers were either secondary or tertial referral centers, and 19 of 20 were central hospitals. One of the centers was a community health care center with special interest in the management of diabetic patients. All attending physicians were either diabetologists or nephrologists and were requested to carefully complete a questionnaire regarding the medical history of the patient; a nurse trained in diabetes or nephrology performed the anthropometric and BP measurements.

Since all central hospitals are referral centers caring for patients with advanced nephropathy and ESRD, a larger proportion of patients with diabetic nephropathy and macrovascular complications were included in the study cohort than had it been an entirely randomly chosen cohort of type 1 diabetic patients. In Finland, patients with diabetic nephropathy are usually referred to the nephrologist at the nearest central hospital when their serum creatinine concentration is around 150 to 200 μmol/l. As can been seen from **Table 9**, the patients with proteinuria originated from 18 of the 20 FinnDiane centers, out of a total of 21 central hospitals in Finland, these patients can be considered representative of a cross-sectional cohort of Finnish type 1 diabetic patients with nephropathy. Furthermore, 184 patients in this study had ESRD. In 2001, according to the Finnish Registry for Kidney Diseases, there were a total of 500 patients with ESRD due to diabetic nephropathy (58). Consequently, this study covered approximately 30% of all ESRD patients in Finland, and since these patients were recruited from 16 of these centers, they are likely to be representative of ESRD patients in Finland (**Table 9**).

Another important issue is whether the type 1 diabetic subjects with normal UAER constituted a representative cohort. An obvious trend in Finland during the last 5 years has been to transfer diabetic patients with no micro- or macrovascular complications from central hospitals to primary health care. If this had already been the case while these patients were being recruited for this study, one could assume that some patients would have remained under central hospital care due to some late complication other than diabetic nephropathy.

Consequently, if they were not true control subjects, they might dilute any potential genetic differences between cases and controls, resulting in a systematic type II error in such an association study (219). However, as can be seen in **Table 9**, only five centers provided no control subjects. These centers had already, except for one small center that recruited only one classifiable patient for this study, transferred the management of uncomplicated type 1 diabetic patients to primary health care, and were thus almost exclusively treating patients with advanced diabetic nephropathy or ESRD. Furthermore, one has to remember the large proportion (45%) of patients ineligible for the study (**Figure 2**) based on their either having too short a duration of diabetes or not being classified due to too few UAERs. However, patients such as these rarely have any late complications, clearly indicating that most of the central hospitals at the time of this study were in charge of all types of adult type 1 diabetic patients regardless of complication status. Selection bias for the control group is thus highly unlikely.

The microalbuminuric group is doubtless the most heterogeneous in this study. Microalbuminuria is considered a strong predictor of overt nephropathy (17-19,285), although this axiom was recently questioned (20). Duration of diabetes should not be neglected, since patients with microalbuminuria and a duration of diabetes more than 20 years

do not accurately predict development of diabetic nephropathy (11). Those with microalbuminuria in this study had an average diabetes duration of 25 years. Consequently, a substantial proportion of these patients are at no stage likely to develop overt nephropathy. It is also noteworthy that none of the positive associations found in this study involved patients with microalbuminuria. This may reflect the heterogeneity of the microalbuminuric group, meaning that those with a short duration of diabetes are genetically more similar to those with diabetic nephropathy in terms of susceptibility genes, whereas those with a long duration are genetically closer to the control group. Unfortunately, since the microalbuminuric group was the smallest of the four groups (n=166), it was impossible to further divide it by diabetes duration.

Classification of the patients into four groups was based on at least two of three recent and consecutive UAER measurements. All centers used timed overnight (nU-Alb) or 24-hour (dU-Alb) urinary collections, but almost all hospitals could provide information from more than the three urinary collections, which further improved the accuracy of the classification. One has to bear in mind that any four groups may not remain entirely constant over time, since both progression and regression may occur within groups, especially for those with microalbuminuria. One 24-hour UAER meas-

urement was therefore performed centrally at the FinnDiane central laboratory and was further compared to the previously measured local UAERs that were the basis for grouping. If this result differed from that of the allocated group, the classification was reassessed including the centrally measured value. Reassessment altered grouping class for only seven patients (0.7%), and these were all patients with microalbuminuria. Significant classification bias is thus very unlikely.

One potential confounding factor regarding patient phenotype is the use of AHT, especially ACE inhibitors. Use of ACE inhibitors became commonplace in the early 1990s after the discovery of their renoprotective effect (41). Is then there a risk that the control subjects (type 1 diabetic patients with normal UAER) would have been genetically microalbuminuric or even proteinuric had they not used AHT? If this were the case, it would create a statistical type II error that would underestimate positive results. However, such a scenario is very unlikely for several reasons. First, only 15% of the control subjects were taking AHT, and only 10% were treated with ACE inhibitors. This is quite similar to the prevalence of hypertension in the non-diabetic background population in Finland (286). Second, control subjects had an average diabetes duration of 30 years. Consequently, if they were genetically susceptible to diabetic nephropathy,

they should already have developed microalbuminuria or overt nephropathy 10 to 20 years before this study, at a time when AHT drugs were not yet commonplace in treatment of diabetic nephropathy (3). Since they had been using AHT for only an average of 8 years, they are likely to represent true control subjects despite their current AHT. Third, for many patients, several historical timed urinary collections were available dating back to the beginning of the 1990s and even to the end of the 1980s with no evidence of elevated UAER. Fourth, each control subject had a normal serum creatinine value. That said, had they escaped a diagnosis of diabetic nephropathy 20 years ago, they should have had the chance to develop impaired renal function during the years up to the time of inclusion in this study, considering the natural history of diabetic nephropathy (3,59).

7.2. Methodological aspects

DNA amplification was performed with PCR, and genotyping with the minisequencing method for all polymorphisms except the Leu7Pro polymorphism in Study III, which used a restriction enzyme according to a previous protocol (133). The minisequencing method provides a numerical value which diminishes the subjectivity of interpretation encountered in regular gel electrophore-

sis in the use of restriction enzymes (271,272). It is sometimes considered a rather laborious, but a highly reliable and a rather efficient method. One prerequisite is a successfully amplified PCR product. That was the case in this study, and genotyping failed in only less than five patients in every study except for the N1077S polymorphism in Study II, where it failed in 35 patients. It is noteworthy that these 35 patients almost exclusively provided the DNA samples drawn between 1994 and 1996, and DNA concentration in these samples was lower than in the majority of samples collected between 1998 and 1999. However, the risk that these patients with low DNA concentrations would have caused genotyping errors in other polymorphisms is unlikely, since PCR failure will technically result in low values in both of the two minisequencing wells, with a default genotyping result instead of a spurious one (271,272). We also evaluated the accuracy of the method in Study I and found its reproducibility to exceed 98%.

The candidate gene method has in general been criticized because it may result in an excessive number of positive associations, often interpreted as type I errors. Commonly, this is seen as a failure of other populations to reproduce such positive associations (219). Furthermore, the tendency to publish only studies with positive results is a well-known phenomenon for all complex

diseases (287,288). New guidelines regarding candidate gene studies have appeared recently in one of the nephrology journals to avoid or at least reduce these problems in diabetic nephropathy (289). These guidelines state that association studies should be published only if the polymorphism is (1) functional, or (2) can be reproduced in another population or in a family-based study, or (3) is corrected for population stratification. Whether these criteria are appropriate remains to be seen.

In this thesis, we studied five genes involved in quite different pathogenetic mechanisms. Interestingly, in all those polymorphisms showing a positive association (Studies I, III, and V) there is evidence of functionality (290-293). On the other hand, regarding the negative Studies II and IV, none of the polymorphisms were functional. It is noteworthy that in these studies a total of three polymorphisms per gene were genotyped. Thus, had there in the near vicinity existed another functional polymorphism important for the development of diabetic nephropathy, it is likely that a positive association would have been found due to LD (294). Thus far, neither the positive nor the negative associations in this study have been confirmed or disputed, due to the short time since publication.

Interestingly, for diabetic nephropathy, the number of published candidate gene studies has increased almost expo-

nentially during the latter half of the 1990s. Thereafter, the number of new studies has, however, diminished, as depicted in **Figure 4**. Regarding most of the genes, there are at most three studies per candidate gene, as shown in the tables in section 2.5. None of the genes in this study had previously been studied in diabetic nephropathy except for the *RAGE* gene, of which two of the three polymorphisms were also investigated in a Danish cohort, with a negative result (255).
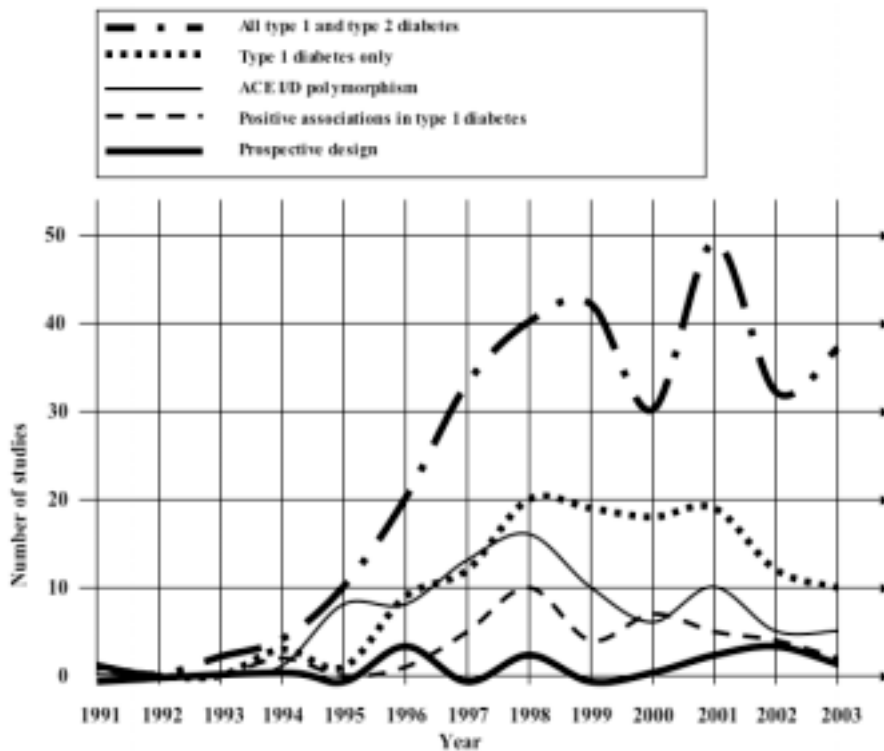

Figure 4. Number of published candidate gene studies since 1991 on gene polymorphism and its association with diabetic nephropathy. Data extracted from PubMed search available at www.ncbi.nlm.nih.gov/. Search performed 15/6/04.

This present study did include only one gene previously studied in diabetic nephropathy. Interestingly enough, the *ACE* I/D polymorphism has been the focus of one-quarter of all candidate genes studies, but the impact of this polymorphism

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is still a matter of debate. Of 27 studies of type 1 diabetic patients with nephropathy involving the *ACE* I/D polymorphism, 13 showed positive associations, and 14 were negative, as seen in **Table 2**. Curiously, a very similar pattern can be seen regarding the same polymorphism in diabetic nephropathy in type 2 diabetes. Recently 40 studies were available on PubMed, and of these, 18 showed positive associations, with 22 negative. The question arises: What could be the reason for such a striking discrepancy and inconsistency? The answer is, of course, unknown, although several plausible explanations can be provided.

First, it is possible that the impact of the *ACE* I/D polymorphism in different populations varies. One of two published meta-analyses showed the I-allele to be protective only in the Japanese population (225), whereas the other showed no differences between any ethnic populations, although the overall impact was stronger in type 1 than in type 2 diabetes (226). Second, it is possible that the polymorphism does not contribute to the initiation of the disease, but instead to its progression, as shown in two recent studies (295,296). Since the number of prospective studies is still modest, the final answer may not yet be known. Many cross-sectional studies may simply not be able to detect a possible difference, due to the heterogeneity of patients with diabetic nephropathy. Third, a major problem is the fact that the inheritance model for diabetic nephropathy is still unknown (188). What cannot be ruled out is whether the impact of the *ACE* I/D polymorphism represents a minor gene effect, but of such a small magnitude that the study populations have thus far been simply too small to reveal any differences between genotypes; hence, the negative studies represent type II errors. Further, one cannot exclude the possibility that the *ACE* I/D is not a causative polymorphism, but rather in LD with another causative gene, as suggested by one American study (297). Consequently, it is possible that due to variations between LD magnitudes in various populations, many studies have failed to detect the association, since the studies have focused only on the I/D polymorphism.

Fourth, the positive associations could also be considered type 1 errors due to population stratification, survival bias, or simply to publication bias (188,287-289). However, one cannot overlook one important observation that favors the *ACE* as a minor risk gene for diabetic nephropathy: All positive published association studies highlight the DD genotype or the D allele as the risk genotype or risk allele. Had the positive associations been spurious, one would expect an approximately equal number of studies in which the II genotype or I allele would be associated with increased risk, since the allele frequencies are very

close to 50%. Consequently, it seems a waste of economic resources to perform a multitude of small studies for one single polymorphism, when a larger number of well-characterized patients should be used in the first place, and further, a prospective design for confirmatory or negative results.

In complex diseases, association studies may be more fruitful if the genes can be chosen from regions pinpointed by linkage studies. In many other complex diseases such as asthma, hypertension, and type 2 diabetes, numerous genome-wide scans have already been performed (298-300). For type 2 diabetes and nephropathy there is only one published genome-wide scan, which shows a suggestive linkage to chromosome 7q35 in the vicinity of the aldose reductase gene (222). However, regarding type 1 diabetes and nephropathy, still no genome-wide scans have appeared. The arduousness of collecting a sufficient number of informative sibling-pairs is certainly one major reason. A long time elapses before the renal status of type 1 diabetic sibling patients can be ascertained, and the mortality for those concordant for diabetic nephropathy is also very high. Rogus *et al.* have, however, suggested using discordant sibling pairs, instead, for cases of diabetic nephropathy (301); this should increase the number of potential siblings considerably.

Can we expect any breakthrough in this field in the near future? Unfortu-

nately, the reproducibility of genome-wide scans has been remarkably low, for example in type 2 diabetes, despite recent improvements (302). Regarding nephropathy in type 1 diabetes, the situation might also be problematic in comparison with many other complex diseases, due to the relatively low genetic relative risk (λ_S-value is 2 to 2.5) (5). It is thus possible that genome-wide scans may be unable at this stage to provide any major help.

7.3. Is there a genetic link between glycemic control and diabetic nephropathy?

Study I showed an association between the functional –374 T/A AA genotype of the *RAGE* promoter and UAER in patients with poor metabolic control. The AA genotype was more common in those with normal UAER than in those with proteinuria. In addition, less CVD occurred in those carrying the AA genotype both in univariate and multivariate analyses. Thus, the AA genotype may protect patients both from proteinuria and from macrovascular complications.

Other data also support the view that altered transcription of the *RAGE* promoter region may affect susceptibility to diabetic nephropathy and CVD. In type 1 diabetic patients, AGE levels are elevated in those with nephropathy and are

highest in those with ESRD (303). Investigation of sites of AGE accumulation in diabetic animals has revealed that increase in RAGE expression by endothelial cells may contribute to more rapid atherosclerosis (304). Infusion of soluble RAGE has further prevented development of vascular disease in mice (92), and RAGE overexpression has been associated with an enhanced inflammatory response in diabetic plaque macrophages (305). Furthermore, when human RAGE is overexpressed in diabetic transgenic mice, the result is glomerular hypertrophy, increased albuminuria, mesangial expansion, advanced glomerulosclerosis, and increased serum creatinine—compared to levels in mice lacking the *RAGE* transgene (90). These studies imply that regulation of the receptor is a crucial event in the pathogenesis of both micro- and macrovascular disease in diabetes. Altered transcription induced by the –374 T/A polymorphism could therefore affect these processes, leading to an altered level of RAGE and a different disease outcome, as is evident in this study.

A linear relationship may exist between HbA_{1c} and increased risk for diabetic complications (29), although a threshold value of 8.1% has also been suggested (30). In our study, an association between AA genotype and protective effect for nephropathy was evident only when HbA_{1c} was higher than 9.5%. A potential caveat in this study was the fact that only 27 patients (8%) with normal UAER had such a high HbA_{1c} value, compared to 91 patients (28%) with proteinuria. Our results do, however, support the hypothesis that the –374 T/A polymorphism affects disease risk in at least a fraction of the patients and that the impact of any possible protective gene is higher when the environmental burden is higher, in this case in those with poor glycemic control.

The results of Study I could be criticized due to the lack of a centrally measured HbA_{1c} value. However, since all centers participated in a national quality assessment program, and since 75% of the centers had identical reference values, this should have no major impact on the study. It is also noteworthy that each center measured HbA_{1c} both in patients with and without diabetic nephropathy. Furthermore, the use of a single—although the most recent—HbA_{1c} value is justified, since one single value offers a valid estimate of average glycemic control during the preceding decade (306). In addition, spurious HbA_{1c} values would perhaps have been more likely to have resulted in a type II error with a dilution of any potential difference, rather than in a type I error. Given this, our results support the view of a gene-environment interaction between *RAGE* and HbA_{1c}, which may in part explain why some patients, despite poor glycemic control, do not develop diabetic nephropathy.

Another interesting potential genetic link between diabetic nephropathy and glycemic control was evident in Study III with respect to the association between HbA_{1c} and the preproneuropeptide Y. Patients heterozygous or homozygous for the Pro7 substitution had a higher HbA_{1c} than did those with the Leu7Leu genotype. The Leu7Pro was also independently associated with HbA_{1c} in multivariate analysis. Analyses were performed both with and without the patients with ESRD, due to a potential survival bias in the ESRD group. The Leu7Pro has earlier been associated in the Finnish population with carotid atherosclerosis, serum LDL cholesterol levels, serum triglyceride levels, and BP (133,247,248). Since these variables are all independent predictors for CVD, and since mortality in ESRD is high in the ESRD group particularly due to CVD (307), a substantial number of patients with ESRD and Pro7 might have been deceased, resulting in a dilution of any potential genetic findings. The prevalence of Pro7 was higher in those with proteinuria than in those with ESRD, although the difference was not significant.

Can the difference in HbA_{1c} between genotypes be explained by impact on insulin sensitivity induced by the Leu7Pro? A recent study in the Finnish population showed no association between Leu7Pro and insulin sensitivity, insulin secretion or glucose metabolism (308). One has to bear in mind, however, that that study was performed in healthy control subjects, whereas our study comprised type 1 diabetic patients suffering various stages of renal complications. In addition, eGDR was lower and the daily insulin dose higher in female patients with the Pro7 substitution, perhaps due to their more pronounced insulin resistance. Moreover, the possible link between the Leu7Pro, insulin sensitivity, and HbA_{1c} is supported by the fact that when the dose was adjusted for HbA_{1c} the association between Leu7Pro genotype and insulin dose disappeared. A potential link may also be the impact of NPY on energy homeostasis. In experimental studies, it appears that elevated NPY expression in the hypothalamus contributes to development of obesity and its related phenotypes (132). This is further supported by the fact that our patients with Pro7 also had a higher BMI. Our results may thus indicate that NPY Leu7Pro is involved in regulation of glycemic control, although the mechanisms call for further investigation.

7.4. The glomerular filter and proteinuria in diabetic nephropathy

Little was known about the glomerular slit-diaphragm until the nephrin protein was described (178) and the nephrin gene (*NPHS1*) cloned (309) a few years

ago. The latter study showed that mutations in the nephrin gene, which lead to a total loss of the nephrin protein from the slit diaphragm, were responsible for the Finnish type of congenital nephrosis in infants already showing massive proteinuria at birth (310). Therefore, in Study II, we hypothesized that common polymorphisms could also lead to a minor disturbance in the nephrin protein, with a subsequent increase in the susceptibility to proteinuria in a diabetic milieu.

No association was, however, apparent between these three polymorphisms in the coding region of the nephrin gene and diabetic nephropathy or any variables related to renal function. The results were also negative when the polymorphisms were analyzed separately, or when genotype combinations were used. On the other hand, this finding does not yet entirely rule out the role of the nephrin gene in the pathogenesis of diabetic nephropathy, since thus far only the coding region and part of the promoter region have been studied. A major impact of the gene on diabetic nephropathy is still unlikely, since the polymorphisms were evenly distributed over the exons. It is thus probable that any possible causative polymorphism in the intron region or promoter region had been in LD with those studied.

Interestingly, the nephrin gene and nephrin protein have been connected in experimental studies with early changes in diabetic nephropathy such as loss of the permeability barrier of the glomeruli (184). Leakage of nephrin into the urine has in a recent human study also been suggested as an early marker of diabetic nephropathy (187). Furthermore, blockade of the AT1 and ACE have reduced nephrin expression in a diabetic milieu (180,182); this may reveal a potential link between the gene and the RAAS. In addition, it is also possible that a deficiency in the podocyte layer complex consisting of nephrin, CD2-associated protein (CD2AP), podocin, and actin cytoskeleton is responsible for functional or morphological changes in the slit-diaphragm in human glomerular diseases other than the Finnish type of congenital nephrosis (186). Although nephrin and CD2AP both bind to actin, it remains unknown known whether nephrin binds directly to actin or whether CD2AP serves as an adapter to this binding site (311). What also cannot be ruled out is whether any yet unidentified proteins are involved in this complex. Data on potential variations in the genes coding these proteins in the podocyte layer are still very scarce, although at least mutations in the podocin gene have been reported to lead to focal segmental glomerulosclerosis (312). In addition, a polymorphism in the caldesmon gene coding the actin cytoskeleton has been associated with diabetic nephropathy (260). It is thus possible in diabetic nephropathy that it is not the nephrin gene itself, but rather the genes regulating the nephrin attachment

to the podocyte layer entity that may contribute to the proteinuric mechanisms.

7.5. The role of dopamine receptors in diabetic nephropathy

The dopamine system is a potential modulator of renal function and BP (125), although current knowledge concerning its influence on humans subjects is scarce (313). Of the five known dopamine receptors, the DRD3 receptor is expressed in the kidney (314). Interestingly, disruption of this receptor in mice has resulted in significant elevation of BP levels and higher renin activity (128). Further experimental data show that nitecapone, an inhibitor of the dopamine metabolizing enzyme catechol-O-methyl transferase, is a potential protective therapy against development of diabetic nephropathy (315). Type 2 diabetic patients with advanced nephropathy have also shown decrease in renal synthesis of dopamine (127).

Study IV uncovered no association between the genotypes or haplotypes of the polymorphisms in the *DRD3* gene and BP levels, kidney function, urinary sodium or potassium excretion or stage of nephropathy, however. Stratifying for gender did not alter the negative results. On the other hand, this study cannot entirely exclude the role of the *DRD3* gene in regulation of BP levels or pathogenesis of diabetic nephropathy, although this seems unlikely for several reasons. First, LD between a causative polymorphism and one of the polymorphisms studied has been probable, especially regarding the promoter region, because of the short distances involved (294). Second, the power in this cohort was sufficient to detect a major or moderate effect. Third, a negative association between the Ser9Gly polymorphism and hypertension has already been reported (316). Other dopamine receptor genes should thus be investigated, because recent data show, in mice, a disruption of the dopamine D2 receptor (DRD2) to result in a sodium-dependent increase in BP (317). Furthermore, two human studies have reported an association between a polymorphism in the coding region of this gene and elevated BP (318,319). In addition, the dopamine D1 receptor (DRD1), another gene among the D1-like receptors, has been associated with hypertension (320).

7.6. Is the *AT2* gene a link between renal function, blood pressure, and gender in diabetic nephropathy?

In Study V, the AA haplotype of the G1675A and A1818T polymorphisms in the *AT2* gene was in male subjects associated with decline in renal function and higher PP; furthermore, the G1675A was also associated with PP in a multivariate

analysis. It is noteworthy that no differences were evident in females between the genotypes or haplotypes and these variables. This finding raises the question whether there is a true gender difference in the pathogenesis of diabetic nephropathy.

Diabetic nephropathy (3) and renal failure in general (217) seem to be more common in males, although epidemiological data are surprisingly scarce regarding this matter. Recent longitudinal data from a Danish cohort supports a male preponderance for diabetic nephropathy, since male sex was an independent predictor for microalbuminuria during that study's 18-year follow-up (218). As can be seen in **Table 8,** the male proportion of our study population is significantly higher in all groups except for those with normal UAER. A similar male predominance can be seen in the entire FinnDiane cohort, which currently consists of more than 4000 type 1 diabetic patients (unpublished data). This finding is also in line with that of the Finnish Registry for Kidney Diseases from 2001, wherein 59% of all patients with renal replacement therapy were males (58). Although these figures support the notion that diabetic nephropathy is more common in males, it is possible that the true proportion of males may be even higher than observed in these studies. Significantly higher cardiovascular mortality is, in fact, a well-known phenomenon in males (216,321),

and male patients with ESRD may have died earlier from CVD; one would therefore have expected to find more females with diabetic nephropathy. In some studies, there seems to be a true male preponderance in diabetic nephropathy, although it is possible that this finding may instead reflect a higher male preponderance in renal disease in general (58), making it not a phenomenon specific only to diabetic nephropathy.

Is the association between AA haplotype and GFR seen in Study V specific for diabetic nephropathy, or does this association reflect a more rapid decline in renal function in general? This study introduced the GFR according to the Cockcroft-Gault formula as a surrogate measure for kidney function for several reasons. GFR is a more sensitive measurement of decline in kidney function than is serum creatinine (269), and it is also more reliable than timed creatinine clearance measurements, which tend to underestimate hyperfiltration and overestimate low GFR (322). Furthermore, timed creatinine clearance is prone to error during urine collection. The use of GFR instead of the four UAER groups in diabetic nephropathy to assess renal complication status may certainly be criticized. However, one has to bear in mind that not all patients with diabetic nephropathy will develop nephrotic albuminuria (323), and that some patients with microalbuminuria will never progress to overt nephropathy, and some

will regress to normal UAER (20). Division into groups according to UAER is also arbitrary, whereas decrease in GFR approaches linearity with a relentless and permanent decline in renal function (324).

We found a positive association with the AA haplotype both when GFR was categorized according to guidelines by the National Kidney Foundation (325) and when GFR was analyzed as a continuous variable. The former analysis enabled inclusion of patients with ESRD, since all ESRD patients must have had a GFR < 60 ml$\cdot$ min$^{-1}\cdot$ 1.73 m^{-2} prior to their renal replacement therapy. The latter analysis excluded patients with ESRD, since a majority had had a renal transplant, and their present GFR would naturally not be informative. The possibility that the AA haplotype is detrimental for renal function in general, independent from diabetic nephropathy, is supported by the fact that no association was evident between the genotypes and UAER, neither when UAER was analyzed as a continuous variable nor when it was categorized into the four UAER groups. Furthermore, we observed no association after adjusting for age between the AA haplotype and retinal laser treatment or macrovascular complications. In addition, no association was apparent between the GFR and any of the polymorphisms in Studies I-IV in a *post hoc* analysis. This supports the fact that the *AT2* gene is perhaps not

involved in the initiation or progression of diabetic nephropathy per se, but rather in the rate of decline in renal function in general.

Interestingly, the AA haplotype of the *AT2* gene was also associated with PP in males, but after adjustment for age not with SBP or DBP. The RAAS is a key player in BP control (326) and also may be involved in the progression of renal disease in diabetes (327). Increase in PP appears to occur 20 years earlier in type 1 diabetic subjects than in non-diabetic subjects, regardless of their renal status, according to our recent study (328). This probably reflects the premature aging of the arterial tree due to hyperglycemia, since the duration of diabetes appeared to be a strong determinant. The impact of the *AT2* gene on BP is supported by studies in non-diabetic subjects, in which positive associations have appeared in males between the *AT2* gene and both hypertension (329) and left ventricular hypertrophy (330,331). It is unclear whether these studies included PP in their analysis. If so, it is still possible that no association would have been detected, since the study subjects were non-diabetic with perhaps a too-slow increase in arterial stiffness for any effect of the *AT2* gene to be detectable. In our study, the 1675A allele was independently associated with PP in males after adjustment for standard risk factors, although the prevalence of hypertension and SBP and DBP did not significantly

differ between the genotypes and haplotypes. Neither was the *AT2* gene associated with CHD after adjustment for age. Impact of the *AT2* gene on CHD may, however, be highest in those with hypertension. In a study of non-diabetic males, the 1675A allele was associated with excess risk among those with systolic hypertension, whereas CHD risk was independent of *AT2* genotype among normotensive individuals (332). Unfortunately, the lack of power in our cohort will make further testing of this hypothesis impossible.

The gender-specific association between the AA haplotype of the X-chromosomal *AT2* gene and renal function and also PP suggests recessive inheritance. If this model is correct, a positive association should have been evident also in comparison of females homozygous for the AA haplotype with those homozygous for the GT haplotype. However, only a non-significant trend toward a higher PP and a decreased GFR occurred in females homozygous for the AA haplotype. This negative association may also be due to the lack of power, since we had only 36 female patients homozygous for the AA haplotype. Thus, the question of whether association with renal function and PP in males is due to the X-chromosomal location of the *AT2* gene, or whether the X-chromosomal location is merely coincidental remains unanswered; it certainly warrants further studies.

7.7. Are there genetic mechanisms in common behind diabetic nephropathy of type 1 and of type 2 diabetes?

This study deals exclusively with the diabetic nephropathy of type 1 diabetic patients. The question does arise whether any of the positive or negative gene associations found can be generalized and considered applicable in type 2 diabetes, as well. Before this question can be answered, it is important to compare not only the clinical phenotype but also the morphological lesions seen in the two disorders. Microalbuminuria in type 1 diabetes is in most cases a clinical sign of incipient diabetic nephropathy (17-19), although its natural clinical course may range from rapid to rather slow progression to ESRD. The morphological lesions are thickened basement membranes (63), mesangial expansion, and narrowing of capillary lumens (64). Ultimately, the glomeruli are destroyed, and the patient develops profuse proteinuria and a relentless decline in renal function. Microalbuminuria can therefore be considered a rather pathognomonic sign of increased risk for overt diabetic nephropathy, although its predictive value regarding the velocity of disease progression may be rather low (11).

Microalbuminuria in type 2 diabetes is a much more complex issue, and is often already present at the time of the diagnosis (333,334). This is hardly ever the case in type 1 diabetes. Moreover, it

is well known that the level of microalbuminuria can remain constant for years in some patients with no signs of progressive renal disease. In such cases, microalbuminuria is usually found without any diabetic lesions in the retina, whereas microalbuminuria in type 1 diabetes is nearly always associated with diabetic retinopathy (112). Interestingly, some proportion of patients with type 2 diabetes also present with a clinical course that resembles that of type 1 diabetic patients, i.e., progressive proteinuria and decline in glomerular filtration rate, as well as other diabetic microvascular complications. Such patients are perhaps genetically similar to those with type 1 diabetes. Such a view is actually supported by the morphological picture of the renal lesions of type 2 diabetes (335). Approximately 30% of the type 2 diabetic patients with microalbuminuria have morphological lesions typical of diabetic nephropathy, as seen in type 1 diabetes. However, another 30% of these patients with microalbuminuria show rather normal kidney morphology despite a degree of microalbuminuria similar to that of the other group. The remaining 40% present rather unspecific lesions such as advanced arteriolohyalinosis, interstitial fibrosis and peculiar glomerular changes, where some glomeruli appear totally normal while others are totally destroyed. Given the complex nature of microalbuminuria in type 2 diabetes, it has been suggested that microalbuminuria in a large proportion of these patients is a result of generalized endothelial dysfunction rather than of renal injury (336).

Microalbuminuria and even proteinuria thus seems to be an important marker not only of advanced diabetic vascular disease but also of nephropathy (337). Consequently, it may be hazardous to try to generalize findings of genetic associations found in type 1 diabetes to all cases of type 2 diabetes. However, it does seem plausible to assume that those with type 1 diabetes and type 2 diabetes with a similar clinical course and similar morphological lesions represent the same disease and the same pathogenetic mechanisms. If this view is true, it also highlights the importance of careful characterization of type 2 diabetes patients for genetic studies.

The next question is whether the genes involved in the pathogenesis of diabetic nephropathy in type 1 diabetes are specific for this trait, or whether the same genes lead to an increase in susceptibility for nephropathy in type 2 diabetes as well, or even in the susceptibility for type 2 diabetes. Further, could genes regulating BP, lipid levels or insulin sensitivity be the common denominators of type 2 diabetes or nephropathy of type 2 diabetes as well as of type 1 diabetes? The *NPY* Leu7Pro polymorphism from Study III has been associated with several variables common for the type 2 diabetic phenotype such as lipids

(133,247,248), BP (247), and even retinopathy (135). Since this polymorphism was associated not only with proteinuria but also CHD, as shown in Study III, it is possible that the common denominators of diabetic nephropathy in type 1 and type 2 diabetes are actually genes that contribute to atherosclerotic mechanisms. This is supported by the fact that in two different patient cohorts, Leu7Pro was also associated with carotid atherosclerosis (247,249).

Furthermore, endothelial dysfunction and premature atherosclerosis may be a consequence of alteration of the *AT2* gene function, as shown in males in Study V. Thus, the increased PP and lower eGDR found in those patients with the AA haplotype may reflect a more rapid atherosclerosis and, although not studied here, this may be similar to the situation in patients with type 2 diabetes. It is thus possible that both the *NPY* and *AT2* genes can be involved in mechanisms contributory to the type 2 diabetic phenotype and to diabetic nephropathy.

On the other hand, certainly genes exist that may not have anything in common with variables constituting the type 2 diabetic phenotype, such as the nephrin gene in Study II. In the case of nephrin, it is interesting that the nephrin gene is also expressed in the pancreas and could possibly affect β cell function (338), although its major function is related to the slit-diaphragm. It is therefore worthwhile to test such genes in

type 2 diabetic patients as well before entirely rejecting them as candidate genes for diabetic nephropathy.

Given the rather large number of candidate gene studies in diabetic nephropathy, the question arises whether there is overlapping of the genes studied for diabetic nephropathy in type 1 and type 2 diabetes. In addition to the genes in Studies II to V, there are at least 17 genes studied only in type 1 diabetes. On the other hand, at least 20 genes are studied exclusively in type 2 diabetes, as can be seen in **Table 19**. Interestingly, in type 2 diabetes, 16 of the 20 genes showed positive associations, whereas only 3 of 17 gave positive associations in type 1 diabetes. Several possible explanations for this striking discrepancy exist. As previously discussed, diabetic nephropathy in type 2 diabetes is a heterogeneous disease (335), and it is possible that a higher number of genes are involved in diabetic nephropathy in type 2 diabetes than in type 1. On the other hand, this lead to the theory that the genes in type 2 diabetes have merely a minor effect; if this is the case, it is questionable whether these studies have had enough power to demonstrate any true positive association, since many of the studies contained a markedly small number of patients. In only two of the studies, in those concerning the matrix metalloprotease 9 gene ($MMP9$) (339,340) and peroxisome proliferative activated agonist receptor gamma gene

Table 19. Genes studied for diabetic nephropathy in only one of the diabetes subtypes

Gene	Potential mechanism	Positive association Reference	No association Reference
Genes studied exclusively in type 2 diabetic patients			
Chymase	BP		(433)
Kallikrein 3	BP	(434)	
Solute carrier family 12 member 3	BP	(435)	
a2b1 integrin	CVD		(436)
Adrenomedullin	CVD	(437)	
G Protein IIIa	CVD		(438)
Lipoprotein lipase	CVD	(439)	
HFE (hemochromatosis)	DIAB	(440)	
Mitochondrial tRNA LeuUUR	DIAB	(441)	
PPARγ2	DIAB	(341,342)	
Glutathione S-transferase M1	GF		(442)
Interleukin-6	GF	(443)	
Matrix metalloprotease 9	GF	(339,340)	
TGF-β stimulated clone 22	GF	(444)	
G*FPT2*	GM	(445)	
Manganese superoxide dismutase	GM	(446)	
Chemotactic cytokine receptor 5	IMMUN	(447)	
CTLA4 (IDDM12)	IMMUN	(448)	
LECAM-1	IMMUN	(449)	
RANTES gene promoter	IMMUN	(447)	
Genes studied exclusively in type 1 diabetic patients			
Alpha adducin	BP		(236)
ATP1 A1	BP		(407)
Epithelial sodium channel	BP		(409)
SA	BP		(414)
von Willebrand factor	CVD		(420)
Werner helicase	CVD		(390)
*ENPP*1 (*PC-1*)	DIAB	(450)	(380,429)
Nuclear factor kappa B	GF		(432)
AGE-R1, AGE-R2, AGE-R3	GM		(255)
RAGE	GM		(255)
Caldesmon	GS	(260)	
Collagen IVA1	GS		(430)
Decorin	GS	(258)	(390)
Complement C4	IMMUN		(421)
Renin	RAAS	(229)	

BP = blood pressure, CVD = cardiovascular disease, DIAB = diabetes, GF = growth factors and cytokines, GM = glucose metabolism, GS = glomerular structure, IMMUN = immunology.

ATP1A = ATPase, Na+/K+ transporting gene, *CTLA4* = cytotoxic T-lymphocyte-associated protein 4 gene, *ENPP1* = alpha 1 polypeptide gene, ectonucleotide pyrophosphatase / phosphodiesterase 1 gene, *GFPT2* = glutamine fructose-6-phosphate transaminase 2 gene, *LECAM-1* = leukocyte-endothelial cell-adhesion molecule 1 gene, *PPARγ2* = peroxisome proliferative activated agonist receptor gamma gene.

(*PPARγ2*) (341,342), could such an association be confirmed in another population. It can be argued that some of the positive associations represent a type I error due to population stratification (343) or a publication bias. It is also quite striking that for none of the genes in type 2 diabetic patients were any contradictory results published; this has been commonplace for association studies in complex diseases and in regard to the I/D polymorphism of the *ACE* gene. It is further interesting that only one conflicting study concerned type 1 diabetic cohorts. Whether there are indeed fewer genes involved in diabetic nephropathy in type 1 diabetes, or whether the frequent positive associations in type 2 are spurious, remains to be elucidated.

7.8. Concluding remarks and future prospects

Genetic factors in diabetic nephropathy have been studied for 15 years (192), but the genetic background of this devastating complication is still far from known. Several major problems complicate the unraveling of the genetics in this disease. It is likely that the λ_S value of 2 to 2.5 in diabetic nephropathy is lower than in many other complex diseases (5). Even the inheritance pattern is still unresolved, since it is not known whether the disease is due to a few major genes or to several minor ones (188). If the latter is true, it is even possible that genes with minor effects have already been studied, but due to negative results they have been denied publication. Successfully published negative results could, on the other hand, result from too-small sample sizes. Furthermore, mortality is still very high in these patients despite intensified treatment and improved prognosis (59). This also makes it very difficult to recruit a sufficient number of sibling pairs to gain enough power for genome-wide scans. However, study of large numbers of type 1 diabetic sibling pairs concordant or discordant for diabetic nephropathy could improve the identification of genomic regions harboring the true susceptibility genes for diabetic nephropathy. Whether there will ever be a sufficient number of informative sibling pairs available from a homogenous population remains to be seen. One of the attempts of the FinnDiane study is to achieve such a goal. Moreover, one has to bear in mind that the genetics of type 1 diabetes and of hypertension, both with higher λ_S values than in diabetic nephropathy, have been studied for a longer period of time, but still the genetics of these traits are also far from unrevealed.

On the other hand, the human genome has recently been sequenced (344,345), giving us reason to expect that the specific functions of many genes will be known within 10 to 20 years. This will make it possible to investigate genes that are entirely new, and at this

stage still unknown that may be important for diabetic complications. Genotyping technology is also advancing with remarkable speed. More than 100 000 genotypes can today be determined faster than could 1 000 when this study was initiated. Computer science and bioinformatics are making rapid progress, and collaboration with biomedicine grows more intense. Of perhaps even greater importance, even larger study populations are available and, above all, they are more carefully characterized than before. The FinnDiane study is one example and is already at this point one of the largest in the world when it comes to studying diabetic complications. International collaboration and networking will be crucial in increasing genetic power still further.

The present study has contributed to our current knowledge by investigating five genes from very distinct biochemical pathways and their potential associations with diabetic nephropathy and other late complications in type 1 diabetes. All studies were association studies, but they can doubtlessly contribute to building strategies for future studies. Polymorphisms in the *RAGE* gene (Study I) could in part explain why some patients despite poor glycemic control do not develop diabetic nephropathy. This gene should be further studied in other populations and especially in a prospective study design. Other AGE-receptor genes should be explored further, and in addition, serum AGEs should be measured and studied in relation to the genetics of the AGE cascade. Polymorphisms in the nephrin gene (Study II) had not been previously studied in diabetic patients. Since it seems unlikely that this gene contributes to diabetic nephropathy, studies should rather be focused on other genes regulating the function of the nephrin protein or other components of the glomerular filter.

In addition to the novel association between HbA_{1c} and the Leu7Pro polymorphism of the preproneuropeptide Y (Study III), these findings support findings indicating involvement with enhanced atherosclerosis. There are, however, still no prospective data regarding the Leu7Pro, and studies in other populations regarding atherosclerosis and its related traits are almost completely lacking. Further functional studies are needed to confirm whether the Leu7Pro itself or another polymorphism in LD is responsible for these findings. Study IV was the first to explore the impact of a gene in the dopamine system with respect to diabetic late complications. The negative association of the DRD3 gene with diabetic nephropathy and BP levels should instead warrant other dopamine receptor studies that may be involved in BP-regulating mechanisms. The *AT2* gene (Study V) had also not been studied previously in diabetic complications. This gene may contribute to the male predominance in diabetic nephropathy

and kidney disease in general, and it may be involved in premature aging of the vessel walls. The impact of this gene needs to be confirmed in prospective studies and in other populations as well, and the function of the gene and its interactions with other genes of the RAAS need to be further clarified.

Doubtless, the results of this thesis are a mere beginning, and the truly important results will be seen when prospective data are available for all candidate genes that show a positive association in a cross-sectional design. However, learning the genetic mechanisms for a disease would be worth nothing if the patient did not gain from this knowledge. It is quite clear that a huge benefit would derive from identifying those at increased genetic risk at a much earlier stage in order to provide these patients with all available treatment to avoid complications. Economic resources should be directed toward these high-risk patients. A majority of them would probably be motivated to further improve their glycemic control, were they only aware of their increased risk profile. Even more importantly, when the genetic mechanisms and pathogenesis of diabetic nephropathy are identified, there will be better prospects for developing entirely new treatment methods. The question is surely not so much *whether* the mystery behind diabetic nephropathy will be solved one day, but rather *when*.

8. MAIN CONCLUSIONS OF THE STUDY

1. Association between the AA genotype of the RAGE –374 T/A polymorphism and CVD as well as albumin excretion in type 1 diabetic patients with poor glycemic control suggests a gene-environment interaction in development of diabetic nephropathy and cardiovascular complications.

2. No support was found in type 1 diabetic patients for involvement of the coding region of the nephrin gene in the pathogenesis of diabetic nephropathy.

3. Leu7Pro polymorphism of the preproneuropeptide Y may contribute to the susceptibility to diabetic nephropathy and coronary heart disease in type 1 diabetic patients, possibly by influencing glycemic control and lipid metabolism.

4. No evidence appeared for involvement of the dopamine D3 receptor gene in pathogenesis of diabetic nephropathy or in blood pressure levels in type 1 diabetes.

5. A gender-dependent association between the *AT2* gene and kidney function as well as between the gene and premature aging of the arterial tree appeared in male type 1 diabetic patients.

9. POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Omkring en tredjedel av samtliga patienter med typ 1 diabetes, som tidigare benämnts ungdomsdiabetes, utvecklar en framskridande njursjukdom som under förloppet av 10-15 år helt förstör njurarna med dialysbehandling eller eventuell njurtransplantation som följd. Dessa patienter innehar en 40-faldigt förhöjd risk för hjärtinfarkt, vilket är orsaken till att mortaliteten är ungefär 50% inom de närmast påföljande 5 åren då dialysbehandlingen påbörjats.

Orsakerna till njursjukdomen är i stort sett okända. En förutsättning för sjukdomen är att blodsockernivån under en längre tid är över det normala. Man vet även att ett förhöjt blodtryck ökar sjukdomsrisken och ger upphov till att sjukdomen framskrider snabbare. Det finns dock patienter som i årtionden haft mycket höga blodsockervärden utan att de utvecklar någon som helst njurskada. Å andra sidan finns det patienter som trots tillfredsställande blodsockerbalans kan utveckla en rätt snabbt framskridande sjukdom. Detta tyder på att genetiska orsaker påverkar om man insjuknar eller inte. Det kanske mest övertygande bevisen för ett genetiskt påbrå är de syskonparstudier, som påvisat att risken för att en person med diabetes kommer att insjukna i njursjudomen, är betydligt högre, om det i samma familj från tidigare finns ett syskon med diabetisk njursjukdom.

Det är uppenbart att diabetisk njursjukdom, i likhet med astma och blodtryckssjukdom, hör till de komplext nedärvda sjukdomarna, där en samverkan mellan omgivningen och den genetiska läggningen bestämmer huruvida man insjuknar och hur snabbt sjukdomen fortskrider. Det har dock visat sig vara mycket mödosamt att hitta genvariationer som antingen utsätter eller skyddar patienten för dylika sjukdomar, eftersom det kan vara fråga om ett stort antal genvariationer vars enskilda verkan är rätt liten. Det finns två huvudstrategier. I den första utgår man ifrån möjligast många syskonpar och genomsöker samtliga kromosomer i hopp om att finna områden som inhyser gener med variationer som kunde tänkas bidraga till sjukdomsprosessen. Denna metod har ännu inte utförts inom diabetisk njursjukdom vid typ 1 diabetes.

I den sk. kandidatgenstrategin utgår man ifrån kända gener vars genvariationer (polymorfismer) kunde påverka sjukdomsmekanismerna. I en associationsstudie jämför man i detta fall huruvida en dylik polymorfism förekommer oftare hos de som har utvecklat njursjukdomen jämfört med en kontrollgrupp bestående av typ 1 diabetiker som

inte har några som helst tecken på njursjukdom trots långvarig diabetes. Denna strategi har används i denna doktorsavhandling, där genpolymorfismer i fem gener undersökts. Beträffande *RAGE*-genen, som kodar en sockerämnesomsättningsreceptor, observerades en association mellan en funktionell polymorfism och en skyddande effekt visavi diabetisk njursjukdom hos patienter med påfallande dålig sockerbalans. I *NPY* genen, som kodar en neurotransmitter, hittades däremot en association mellan en funktionell polymorfism samt såväl diabetisk njursjukdom, triglyceridhalt, sockerbalans, som kransartärsjukdom. I *AT2* genen, som befinner sig på X-kromosomen hittades en association mellan en polymorfism och njurfunktionenen samt blodtrycket hos män, vilket betyder att genen eventuellt delvis skulle kunna förklara varför njursjukdomen är vanligare hos män. Två av generna i denna studie uppvisade däremot inga samband alls med någon av de undersökta variablerna.

Det bör avslutningsvis poängteras att samtliga positiva associationer ännu kräver uppföljningsstudier för att man skall kunna dra några definitiva slutsatser angående orsak och verkan. Om de genetiska orsakerna till diabetisk njursjukdom skulle identifieras, kunde detta möjliggöra ett tidigt ingripande och en fokusering av de ekonomiska resurserna på just de patienter som har gener som utsätter dem för sjukdomen. Detta kunde på sikt leda till att sjukdomen i framtiden helt kunde förebyggas eller åtminstone uppskjutas.

10. YLEISTIETEELLINEN YHTEENVETO SUOMEKSI

Kolmasosalle kaikista tyypin 1, eli nuoruustyypin diabetesta sairastaville potilaille, kehittyy etenevä munuaistauti, joka 10-15 vuodessa tuhoaa munuaiset ja johtaa potilaan dialyysihoitoon tai munuaisensiirtoon. Näillä potilailla on 40-kertainen riski sairastua sydäninfarktiin ja heidän kuolleisuutensa dialyysihoidon alkamisajankohdasta lähtien on noin 50% viidessä vuodessa.

Diabeettisen munuaistaudin perimmäiset syyt ovat yhä tuntemattomat. Ehkä tärkein syy sairastumiseen on pitkäaikainen normaalia korkeampi verensokeritaso. Lisäksi tiedetään että korkea verenpaine lisää sairastumisriskiä ja nopeuttaa taudin etenemistä. On kuitenkin potilaita, joilla on vuosikymmeniä ollut hälyttävän korkeita verensokeriarvoja ilman että munuaisiin kehittyy minkäänlaisia vaurioita, ja toisaalta potilaita, jotka varsin hyvästä verensokeritasapainosta huolimatta kehittävät nopeasti etenevän munuaistaudin. Tämä viittaa vahvasti siihen, että perintötekijöillä on osuutta sairastuvuuteen. Vahvimmin perintötekijöiden osuutta tukevat todisteet löytyvät tutkimuksista, joissa osoitetaan diabetesta sairastavalla nuoremmalla sisaruksella olevan kohonnut riski sairastua munuaistautiin, jos vanhemmalla sisaruksella on jo todettu tämä tauti.

On selvää, että diabeettinen munuaistauti, samoin kuin esimerkiksi astma ja verenpainetauti, kuuluvat nk. monitekijäisiin tauteihin, joissa ympäristövaikutusten ja perintötekijöiden vuorovaikutus yhdessä määräävät sairastuvuuden ja taudin etenemisnopeuden. Koska taudin taustalla voi olla useita altistavia perintötekijöitä joissa yksittäisen tekijän vaikutus on pieni, on osoittautunut erittäin vaikeaksi löytää sekä potilaita altistavia että suojaavia perintötekijöitä. Kyseisten tekijöiden löytämiseksi on olemassa kaksi päästrategiaa. Näistä ensimmäisessä tutkitaan potilaiden perimää mikrosatelliitti-markkereilla jotta voitaisiin löytää sairaudelle altistavat kromosomaaliset alueet. Tällaista koko genomia kattavaa analyysiä ei vielä ole tehty tyypin 1 diabeettista munuaistautia sairastavilla potilailla.

Ehdokasgeenimenetelmässä lähtökohtana puolestaan on tutkia jo aiemmin tunnistettuja, mahdollisesti munuaistautiprosessin laukaiseviin tekijöihin vaikuttavia geenivariaatioita. Tällä menetelmällä tutkitaan esiintykö jokin geenivariaatio useammin diabeettistä munuaistautia sairastavilla potilailla kuin kontrolliryhmällä, joka koostuu diabetespotilaista, joilla ei ole minkäänlaisia munuaistaudin merkkejä taudin pitkästä kestosta huolimatta. Tässä tutkimuksessa tutkittiin viiden geenin variaatioita. Sokeriaineenvaihduntareseptoria koodaavassa *RAGE*-geenissä todettiin yhteys (assosiaatio) erään toiminnallisen vari-

aation ja diabeettiselta munuaistaudilta suojaavan vaikutuksen valilla niilla potilailla, joilla oli erityisen huono verensokeritasapaino. *NPY*-geenissa, joka koodaa erasta hermovalittajaainetta, loydettiin yhteys toiminnallisen variaation seka diabeettisen munuaistaudin, triglyseridipitoisuuden, verensokeritasapainon seka sepelvaltimotaudin valilla. X-kromosomissa sijaitsevan *AT2* geenin todettiin assosioituvan toiminnalliseen geenivariaatioon seka munuaistoimintaan ja verenpainetasoon miehilla, mika voisi viitata nimenomaan miesten korkeampaan sairastuvuuteen valtimokovettumatautiin. Kahden geenin kohdalla ei

loydetty minkaanlaista assosiaatiota tutkittuihin tekijoihin.

Lopuksi on korostettava, etta kaikki todetut assosiaatiot vaativat seurantatutkimuksen mahdollisten syy-seuraussuhteiden varmistamiseksi. Diabeettisen munuaistaudin taustalla olevien perintotekijoiden loytyminen mahdollistaisi varhaisen puuttumisen tautiprosessiin seka taloudellisten voimavarojen kohdentamisen juuri niihin potilaisiin, joilla on munuaistaudille altistavat perintotekijat. Tallóin taudin puhkeaminen voitaisiin estaa tai ainakin sen etenemista voitaisiin hidastaa.

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