Factors Influencing the Risk of Diabetic Nephropathy
- Analyses of Genes, Smoking and Diet

Anna Möllsten

Department of Clinical Sciences, Paediatrics
Umeå University, Sweden
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Cover art:

Science Photo Library / Computer artwork of a DNA molecule. DNA (deoxyribonucleic acid) is composed of two strands (silver outside) twisted into a double helix. Each strand consists of a sugar phosphate backbone attached to nucleotide bases (blue spheres). There are four bases: adenine, cytosine, guanine and thymine. The bases are joined together by hydrogen bonds (silver, between spheres). DNA contains sections called genes that encode the body's genetic information.
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Abstract

Diabetic long-term complications, despite intensive treatment, cause serious handicaps at relatively young age in diabetic patients. Diabetic nephropathy (DN) develops in up to 30% of patients with type 1 diabetes (T1D). Besides the eventual loss of kidney function, with need for dialysis treatment and transplantation, this complication also increases the risk of early death from cardiovascular disease. In addition to hyperglycaemia, the risk of developing DN is influenced by a number of life-style related factors, such as smoking and diet, but the mechanisms of action of these factors are largely unknown. The incidence of DN is not linearly related to diabetes duration. There is a peak incidence of DN at 15-20 years and this, together with results from family studies, shows that genetic factors are important contributors. Possible candidate genes are those involved in regulation of intraglomerular pressure and blood pressure, oxidative stress and inflammation.

The main aims of this thesis were:

● To investigate the risk of DN associated with polymorphisms in;
  A) the endothelial NO-synthase gene (NOS3) and genes in the renin-angiotensin-system (RAAS) (all involved in the regulation of intraglomerular pressure).
  B) the manganese superoxide dismutase gene (SOD2) (involved in the regulation of oxidative stress).
  C) the ICAM1 gene (involved in activation and migration of lymphocytes)

● To investigate gene-smoking interactions

● To investigate the influence of normal diet on risk of microalbuminuria.

The aims were addressed in different case-control settings, including 347 T1D patients from Sweden and 1163 patients from Finland, with or without DN, defined as; overt DN – having albumin excretion rate (AER) ≥200 μg/min, incipient DN – AER between 20 and 200 μg/min, non-DN controls – having AER <20 μg/min and at least 20 years of diabetes duration. In one study also non-diabetic healthy individuals were included to asses the risk of T1D associated with the ICAM1 gene.

Results: The RAAS genes were investigated in the Swedish sample set and there was an association between a polymorphism in the angiotensin II type 1 receptor (AGTR1) gene and overt DN, when adjusting for age, duration
of diabetes, HbA1c, sex and smoking (adjusted OR=3.04, 99% CI=1.02-9.06). Also a synergistic interaction with smoking was indicated.

The ICAM1 gene was investigated in the Swedish sample set, but no association with DN was found. There were, however, associations between T1D and two polymorphisms in this gene, rs281432 (OR=1.64, 95% CI=1.14-2.38) and rs5498 (OR=2.46, 95% CI=1.59-3.80).

In the combined Swedish/Finnish sample set, the Glu/Glu genotype of the Glu298Asp polymorphism in the NOS3 gene was associated with DN when age at diabetes onset, duration of diabetes, HbA1c, blood pressure, sex and smoking were taken into account (adjusted OR=1.46, 95% CI=1.12-1.91). There was also association between a polymorphism in the MnSOD gene and DN in this sample set. Homozygosity for the valine-allele of the Val16Ala polymorphism was associated with increased risk of DN in a model including age at diabetes onset, duration of diabetes, HbA1c, sex and smoking (adjusted OR=1.32, 95% CI=1.00-1.74).

Smoking was associated with DN (OR=2.00, 95% CI=1.60-2.50) and in the Swedish sample set there were indications of interactions between smoking and the NOS3 and SOD2 genes, but these results could not be confirmed in the Finnish sample set.

A high protein intake can enhance glomerular filtration rate and accelerate progression to DN, also other dietary components such as fat, fibres, vitamins and the ratio red/white meat have been discussed as important for DN development. In a nested case-control study including young T1D patients, the normal dietary intakes of protein and other nutrients were assessed using a semiquantitative questionnaire. The results showed that T1D patients consuming more than 6.5 g fish protein (>75th percentile) per day were at slightly lower risk to have microalbuminuria in both crude (OR=0.49, 95% CI=0.25-0.97) and adjusted analyses (OR=0.26, 95% CI=0.09-0.76, adjusted for age, duration of diabetes, sex, HbA1c, mean arterial pressure, BMI, region, smoking, energy intake and fish fat intake).

**Conclusions:** The risk of having diabetic nephropathy is influenced by at least two genes controlling blood pressure and one gene protecting against oxidative stress. Smoking also increases the risk of DN and our findings indicate that smoking may accentuate the effect of the AGTR1, NOS3 and SOD2 genes. Normal dietary intake of protein was not associated with risk of having microalbuminuria in young T1D patients, on the other hand, an intake of fish protein above the 75th percentile decreased the risk of microalbuminuria.
List of Original Papers

This thesis is based on the following articles and manuscripts, which will be referred to in the text by their Roman numerals (I-VI). The published papers have been reprinted with permission from the publishers.


Abbreviations

Abbreviations frequently used in the thesis
ACE – angiotensin converting enzyme
AER – albumin excretion rate
AGE – advanced glycation endproduct
AGT – angiotensinogen
AGTR1 – angiotensin II type 1 receptor
CI – confidence interval
CYP11B2 – gene encoding aldosterone synthase
DAG – diacylglycerol
DN – diabetic nephropathy
ECM – extracellular matrix
eNOS – endothelial cell nitric oxide synthase
ESRD – end stage renal disease
GFR – glomerular filtration rate
HbA1c – glycosylated haemoglobin
ICAM-1 – intercellular adhesion molecule-1
MnSOD – manganese superoxide dismutase
NO – nitric oxide
NOS3 – the gene encoding eNOS
OR – odds ratio
PA – plasminogen activator
PAI-1 – plasminogen activator inhibitor-1
PKC – protein kinase C
RAAS – renin-angiotensin-aldosterone system
RERI – relative excess risk due to interaction
ROS – reactive oxygen species
SNP – single nucleotide polymorphism
SOD2 – the gene encoding MnSOD
T1D – type 1 diabetes
T2D – type 2 diabetes
TGF-β – transforming growth factor-β
VEGF – vascular endothelial growth factor
Introduction

Type 1 diabetes (T1D) is caused by autoimmune destruction of the insulin producing β-cells. These cells are located in clusters in the pancreas called the islets of Langerhans. Destruction of the β-cells results in total lack of insulin and chronic hyperglycaemia. Over the years with diabetes there is a risk of developing severe long-term complications due to pathology in small and large blood vessels, micro- and macroangiopathy. Examples are diabetic retinopathy (pathology of the capillaries in the retina), nephropathy (kidney damage) and cardiovascular disease, like stroke and coronary heart infarction. Diabetic nephropathy (DN) is the most serious long-term complication, leading to renal failure and end stage renal disease (ESRD), with dialysis and transplantation as only treatment. DN also leads to increased mortality caused by cardiovascular disease (1, 2). Improved long-term glycaemic control has reduced, or delayed, the risk to develop DN in diabetic patients (3) but still the prevalence at 25-30 years of duration is 10-30% (4, 5).

There are complex combinations of genetic, environmental and lifestyle related factors involved in the development of DN and there are many different mechanisms by which hyperglycaemia can cause diabetic complications. It is important to find patients at high risk as early as possible and initiate preventive treatment to avoid, or at least postpone, the development of DN. Some of the genetic and lifestyle related factors involved in the development of DN are investigated in this thesis.

The Kidneys

The kidneys have a range of functions and one of the most important is excreting waste products (mainly urea from protein digestion). They are also responsible for maintaining water and salt balance in the body, keeping the correct pH and regulating the blood pressure.

Structure and haemodynamics of the healthy kidneys

The functioning units of the kidneys are called nephrons and there are between 1 and 3 million nephrons in each kidney. The nephron consists of
three main parts, the glomerulus, Bowman’s capsule and the tubule. The glomerulus is a bundle of capillaries in close contact with the glomerular basement membrane. The blood vessel leading blood into the glomerulus is the afferent arteriole and the vessel leading out is the efferent arteriole. The afferent arteriole is wider in diameter than the efferent, causing a high pressure in the glomerulus when more blood is allowed in than out. The outside of the glomerular basement membrane is covered with podocytes (glomerular visceral epithelial cells) and their foot processes. They are attached to the membrane with different adhesion molecules. Adjacent podocytes and foot processes are separated by narrow spaces, filtration slits. The excess pressure inside the capillaries forces plasma through the capillary walls and through the filtration slits. The last barriers that keep the plasma proteins in the blood and let water and ions pass through are porous membranes called slit diaphragms, which are relatively impermeable to plasma proteins. Through this filtration, primary urine is formed. Surrounding the glomerular capillaries is Bowman’s capsule, a hollow ball of epithelial cells which forms the urinary space. When the primary urine leaves the urinary space of Bowman’s capsule it enters the tubular proportion of the nephrons, where nutrients and minerals are reabsorbed from the primary urine, waste products and toxins are secreted, and water is secreted or retained depending on the hydration status of the body (6).

How much blood that is filtrated each minute, the glomerular filtration rate (GFR), is controlled by the diameters of the afferent and efferent arterioles, the hydrostatic pressure in the urinary space and the osmotic pressure of the blood. The GFR also depends on the size of the person. For a healthy individual the GFR is about 70 ml of blood each minute per m$^2$ of body surface (6). Different hormones and signals can control the dilation and constriction of the arterioles, for example the renin-angiotensin-aldosterone system (RAAS) and the vasodilating molecule nitric oxide (NO).

**Haemodynamics of the kidneys during diabetes**

In a hyperglycaemic environment the kidneys are affected in several ways. Reabsorption of glucose from the urine is normally very effective, but during hyperglycaemia all the glucose can not be absorbed and is instead
secreted. Water is also eliminated due to osmotic effects, resulting in large urine volumes and dehydration.

It has been shown that patients with elevated GFR are at increased risk of developing DN (7, 8). During the years with diabetes, the haemodynamics of the kidneys and the GFR mostly depends on metabolic control and blood pressure. Before insulin treatment is started the GFR is increased (9), but it is normalized with the initiation of insulin treatment (fig. 1a). However, the GFR remains slightly elevated in at least 25% of the T1D patients, even if it is within normal range (10, 11), it is likely that these patients have a higher risk of developing DN later on. The GFR can be temporarily increased during periods of poor metabolic control but this can be reversed by effective insulin treatment (12, 13). Increased blood pressure also increases the GFR (14, 15) and if antihypertensive medication is used, the rate of decline in kidney function can be markedly reduced (16, 17).

The albumin excretion rate (AER) is at first affected the same way as the GFR. The AER is increased at the onset of diabetes, but when insulin treatment normalizes the GFR also the AER is normalized (fig. 1b). Albumin leakage into the urine is a well-known early marker for the development of DN and also the first clinical sign of kidney damage (7, 18). If DN progresses, GFR slowly decreases due to the reduced filtration surface while albumin leakage increases. At first there will be relatively small amounts of urinary albumin, microalbuminuria (also called incipient nephropathy). As the kidney damage progresses the amount of albumin increases and the patient develops macroalbuminuria (overt nephropathy). Albuminuria is also a marker for other complications such as proliferative retinopathy and cardiovascular complications (19). With decreased GFR, hypertension will develop, which in turn accelerates the process (20).

During the development of DN there are morphological changes in the glomeruli, for example thickening of the glomerular basement membrane, expansion of the mesangium and accumulation of extracellular matrix (ECM). The leakage of albumin per se accelerates the progression of kidney disease, probably because albumin accumulates in the mesangium and the extracellular structures in the glomeruli, which may stimulate cell proliferation and accumulation of ECM (21). There is also loss of podocytes due either to apoptosis or cell detachment, or both (22), which leads to increased permeability for albumin molecules. Later on there is
sclerosis of the glomeruli which reduces the filtration surface (23, 24) and the reduced capability of some glomeruli to filtrate the blood increases the workload for the others, damaging also the remaining glomeruli.

**Figure 1:** a) How GFR changes with duration of diabetes. Due to hyperglycaemia GFR is increased at the onset of diabetes, but with insulin treatment GFR is reduced to normal or near normal values. Patients who have higher GFR are over the years at higher risk of developing DN. Hypertension can accelerate the course of the disease and treatment with antihypertensive medication can decrease the rate of DN progression. b) How AER changes with duration of diabetes, pictures adapted from Mogensen 1983 (25).
Disease Mechanisms

Hyperglycaemia is associated with glomerular hyperfiltration during the early stages of diabetes, which can lead to glomerulopathy because of the effects of increased intraglomerular pressure (8). These effects can be mediated through systems controlling systemic and intra-renal blood pressure and blood flow, such as the renin-angiotensin-aldosterone system (RAAS) and nitric oxide (NO). Hyperglycaemia is also known to induce oxidative stress, which has been suggested as a contributor in the development of diabetic complications, including DN (26). Since increased blood glucose level leads to an overloaded glycolysis, it alters the glucose metabolism, activates the polyol and hexosamine pathways and increases the formation of advanced glycation endproducts, AGEs, through non-enzymatic glycation of proteins. Also lifestyle related factors like diet and smoking are important in the development of DN. A summary of the different effects of hyperglycaemia is shown in figure 2.

Why are the genes interesting?

The development of diabetic nephropathy does not only depend on the diabetic milieu because not all T1D patients are affected. The incidence of DN peaks in the second decade of disease and after 25 years the risk of developing the disease is much decreased, in contrast to other diabetic complications where the risk increases with duration time. Thus, there seem to be a subgroup of patients who are susceptible, probably from genetic predisposition, and they develop the disease during the first two decades of diabetes (1, 4, 5, 27). There are also studies showing familial clustering of DN (28, 29), which strongly suggests involvement of genetic factors.

Blood pressure regulation

The renin-angiotensin-aldosterone system

An important factor in the development of DN is the regulation of blood pressure. The RAAS is very much involved in this process and this system depends on several steps (fig. 3). The enzyme renin is secreted from the juxtaglomerular cells and cleaves angiotensinogen (AGT) to form angiotensin I. Angiotensin converting enzyme (ACE) then turns the inactive...
**Introduction**

Genetic factors, eNOS, RAS, ROS

↑ AGEs, hexosamine- and polyol-pathways

↑ PKC, TGF-β, VEGF and PAI-1.

Cellular injury, matrix overproduction, glomerular sclerosis

Diabetic Nephropathy

**Figure 2:** Summary of suggested mechanisms associated with diabetic nephropathy. Hyperglycaemia alters the glucose metabolism, increases AGE formation, activates the polyol and hexosamine pathways, affects NO-synthase, the RAAS and the formation of reactive oxygen species (ROS). Taken together this leads to hyperfiltration, activation of growth factors (TGF-β, VEGF) and other signals (PKC), cellular injury, production of extracellular matrix and sclerosis, causing diabetic nephropathy. Subsequent development of hypertension will accelerate the process (20). Contributing factors can be smoking and diet.

Angiotensin I into the active octapeptide angiotensin II, which is now ready to interact with specific receptors. There are two known types of angiotensin II receptors, type 1 (AGTR1) and type 2 (AGTR2). Both types of receptors are present in the adrenal cortex and kidney, but AGTR1 dominates in the vascular smooth muscle cells and exerts most of the functions of angiotensin II (30). When binding to its receptor, angiotensin
II increases the intraglomerular pressure by causing vasoconstriction in the renal vessels via stimulation of different signals in the afferent and efferent glomerular arterioles (31). Angiotensin II decreases salt and water secretion via the kidneys and this also adds to the rise in blood pressure.

Figure 3: Angiotensinogen is processed by renin to angiotensin I, which is activated by angiotensin converting enzyme to angiotensin II, then binding to the AGTR1 on the target cells. ACE also degrades bradykinin.

ACE inhibitors and AGTR1 antagonists have more beneficial effects, besides lowering blood pressure, and are more efficient in treatment of DN than other antihypertensive medicines (32-34). ACE not only converts angiotensin I to angiotensin II, it also degrades the vasodilator bradykinin (fig. 3). Results from animal studies suggest that this can be one of the reasons why ACE inhibitors have particularly positive effects on renal function in patients with diabetes (35). Another feature of angiotensin II is
that it induces transforming growth factor-β1 (TGF-β1) and thereby stimulates mesangial growth and production of extracellular matrix (ECM) (36-39), which contributes to DN development. It also induces production of oxygen radicals, at least in vitro (40). In addition, the angiotensin II molecule stimulates the production of the mineralcorticoid hormone aldosterone, which increases the blood pressure by increasing salt re-absorption (41). When using a rat-model, Brown et al. found that an aldosterone antagonist decreased the level of plasminogen activator inhibitor-1 (PAI-1), which was correlated to the degree of sclerosis in the kidney (42). In treatment of patients with DN, aldosterone antagonists have been suggested as a complement to ACE inhibition (43) and more evidence is gathering that the effect of aldosterone on renal injury is independent of blood pressure (44).

**Genes of the renin-angiotensin-aldosterone system**

Family studies have shown that there is higher prevalence of hypertension among non-diabetic parents of T1D probands with DN, compared to parents of normoalbuminuric T1D patients (45-48), so susceptibility genes proposed for hypertension can be of importance in the development of this complication. Hence our thoughts go to the RAAS and its obvious association with DN. Mutations in any of the genes involved in the RAAS can lead to changes in the blood pressure control system.

A number of genetic markers within the RAAS have been examined. **Angiotensinogen:** The M235T polymorphism (rs699) is a single nucleotide polymorphism (SNP) in the angiotensinogen gene (30) that has been related to essential hypertension and albuminuria (49). The M235T polymorphism gives an amino acid exchange in position 235, where a methionine has been exchanged for a threonine. Another SNP, in the promoter region of the angiotensinogen gene, has been suggested to influence the plasma level of angiotensinogen and also the blood pressure. This polymorphism is located at position –20 and is an A-C exchange (A-20C). When there is a C in position –20 the transcription from the angiotensinogen promoter seems to be slightly elevated (50). This could lead to increased levels of angiotensinogen, and possibly contribute to essential hypertension, thereby also making the polymorphism interesting in the development of DN.
**ACE:** Plasma levels of the important enzyme ACE are under genetic control. The insertion/deletion (I/D) polymorphism (rs13447447), in intron 16 of the ACE gene, is observed as the presence or absence of a 287 bp long sequence. This polymorphism has noticeable impact on the plasma levels. Deletion homozygous individuals have the highest levels of plasma ACE, heterozygous individuals have intermediate levels, while individuals homozygous for the insertion have the lowest levels of plasma ACE (51). Results from many association studies have been unambiguous, but a meta-analyses by Fujisawa et al., 1998, and Ng et al., 2005, showed that the D allele was significantly associated with DN (52, 53). Clinical studies have proven that ACE inhibitors can retard the development of DN (32, 54, 55) and it is also known that the production of ACE is down-regulated in the presence of insulin (56), which can lead to elevated levels of ACE in an insulin depleted environment.

**AGTR1:** As well as ACE, the AGTR1, mediating the angiotensin II signal into the cell, is a target for antihypertensive medications. An AGTR1 antagonist has been shown to have more beneficial effects on the cardiovascular system than just lowering the blood pressure (33). A base pair exchange in position 1166, where an adenosine has been exchanged for a cytosine (A1166C, rs5186) has been associated with hypertension in non diabetic subjects (30). The C-allele has also been associated with increased blood pressure as a response to hyperglycaemia in T1D patients (57). Doria et al. found a synergistic effect of the C-allele and hyperglycaemia increasing the risk of DN in T1D patients (58), but this finding could not be repeated in later studies (59).

**Aldosterone:** The hormone aldosterone is produced by aldosterone synthase, encoded by CYP11B2 (60). In the promoter region of CYP11B2, a SNP (C-344T, rs79998) is located in a binding site for steroidogenic transcription factor-1 (SF-1). The T-allele has been associated with higher aldosterone excretion rate (61) and with hypertension (61-64), which makes it a possible candidate for contribution to DN risk. However, the T-allele was not associated with DN in Danish T1D patients (65).

**Nitric oxide and nitric oxide synthase**
Nitric oxide (NO) is an intercellular messenger molecule, with vasodilating effect. It is involved in the regulation of vascular tone and the glomerular
INTRODUCTION

haemodynamics. Macrophages use NO to kill invading bacteria (66) and in medicine NO is used for example in treatment of angina pectoris. NO is produced from L-arginine by the enzyme nitric oxide synthase (NOS) (67). NOS exist in three different isoforms, neuronal, inducible and endothelial (nNOS, iNOS and eNOS), encoded by different genes and regulated differently (NOS1, NOS2 and NOS3). The production of NO in endothelial cells (by eNOS) is important for the regulation of the systemic blood pressure (68, 69) as well as the intraglomerular pressure (70). Nitric oxide decreases the blood pressure by relaxing the blood vessels, but at the same time it may increase the blood flow, therefore the regulation of NO production is important for maintaining the delicate balance of the glomerular blood flow. In experimental diabetes there is increased eNOS content in the afferent arterioles of the glomeruli, but normal eNOS content in the efferent arterioles (71). This can lead to increased blood flow into the glomeruli but unchanged outflow, inducing glomerular hyperfiltration and contributing to the risk of DN development.

A polymorphism in intron 4 of the NOS3 gene, consisting of four or five 27 bp repeats (designated a and b respectively) has been associated with variations in plasma levels of NO metabolites, but there are some contradictory results. Wang et al. found that a-homozygous subjects had elevated levels of NO metabolites compared to b-homozygous or heterozygous subjects, in a study with participants with European origin (72). On the other hand, in a study from Tokyo Tsukada et al. found that b-homozygous subjects had higher levels of NO-metabolites (73). These discrepancies are probably due to differences between the ethnic groups. It has been found, however, that the a-allele is more frequent among patients with DN (74, 75) and also among patients with non-diabetic renal disease (76). The seemingly contradictory results that the a-allele is associated with increased amounts of NO (in European subjects) and at the same time associated with DN, emphasizes that NO has different effects in different organs. The vasodilating effect decreases the systemic blood pressure, but when produced in the kidney it causes an increased blood flow and thus increases the intraglomerular pressure, which could initiate renal damage. When studying smoking patients Wang et al. found that individuals homozygous for the a-allele had an increased risk of coronary artery disease.
which indicates that there can be environmental factors enhancing the effect of this gene.

In exon 7 of \textbf{NOS3} there is a SNP that results in a Glu-Asp amino-acid exchange in position 298 (rs1799983). This amino-acid exchange has been associated with cardiovascular symptoms like essential hypertension and myocardial infarction (78, 79). Also ESRD and type 2 diabetes-derived ESRD have been associated with this polymorphism in Japanese populations (80, 81). In American T1D patients, however, no association with DN could be found (75). Summaries of previous DN association studies are shown in tables 1a and 1b.

\textbf{Table 1a:} Previous studies on association between DN and the \textbf{NOS3} Glu298Asp polymorphism.

<table>
<thead>
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<th>1st author, year</th>
<th>Diabetes</th>
<th>n</th>
<th>Country</th>
<th>Result</th>
<th>Ref.</th>
</tr>
</thead>
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<td>Zanchi, 2000</td>
<td>T1D</td>
<td>347</td>
<td>USA (Caucasian)</td>
<td>No association</td>
<td>(75)</td>
</tr>
<tr>
<td>Cai, 1998</td>
<td>T2D</td>
<td>400</td>
<td>Australia (Caucasian)</td>
<td>No association</td>
<td>(82)</td>
</tr>
<tr>
<td>Shin Shin, 2004</td>
<td>T2D</td>
<td>177</td>
<td>Korea (Korean)</td>
<td>Asp-allele associated with progression of DN</td>
<td>(83)</td>
</tr>
</tbody>
</table>

\textbf{Table 1b:} Previous studies on association between DN and the \textbf{NOS3} NOS4ab-polymorphism.

<table>
<thead>
<tr>
<th>1st author, year</th>
<th>Diabetes</th>
<th>n</th>
<th>Country</th>
<th>Result</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zanchi, 2000</td>
<td>T1D</td>
<td>347</td>
<td>USA (Caucasian)</td>
<td>Association with a-allele</td>
<td>(75)</td>
</tr>
<tr>
<td>Neugebauer, 2000</td>
<td>T2D</td>
<td>215</td>
<td>Japan (Japanese)</td>
<td>Association with a-allele</td>
<td>(74)</td>
</tr>
<tr>
<td>Degen, 2001</td>
<td>T1D/T2D</td>
<td>324/414</td>
<td>Germany (Caucasian)</td>
<td>No association</td>
<td>(84)</td>
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<tr>
<td>Shimizu, 2002</td>
<td>T2D</td>
<td>433</td>
<td>Japan (Japanese)</td>
<td>No association</td>
<td>(85)</td>
</tr>
<tr>
<td>Rippin, 2003</td>
<td>T1D</td>
<td>1720</td>
<td>UK (Caucasian)</td>
<td>No association</td>
<td>(86)</td>
</tr>
</tbody>
</table>
Glucose metabolism

The polyol and hexosamine pathways

In case of hyperglycaemia all glucose cannot be metabolised by the glycolysis. This stimulates the polyol and hexosamine pathways, which are alternative pathways for the glycolytic products. In the polyol pathway the enzyme aldose reductase turns toxic aldehydes into inactive alcohols (87) but during hyperglycaemia the polyol pathway is also stimulated to produce sorbitol and fructose (fig. 4). Sorbitol is not normally produced in large amounts and does not diffuse out of the cells, causing an increase in intracellular fluids, followed by complex biochemical changes including decrease in Na\(^+\)/K\(^+\)-ATPase activity and NADPH. Normally the accumulation of sorbitol is a way to withstand osmotic stress (88) but the high amounts of glucose in diabetes can make the sorbitol accumulation toxic. In this process there is a risk that the cells become depleted of NADPH. NADPH acts as a cofactor for glutathione reductase, which reduces oxidized glutathione (GSSG) to the intracellular antioxidant reduced glutathione (GSH) (89). Oxidative stress increases in the cells because of the reduction in NADPH. Oxidative stress, in turn, activates aldose reductase via toxic aldehydes formed during lipid peroxidation (90, 91) (fig. 4). In addition, inhibition of aldose reductase is known to prevent glucose induced increase of protein kinase C (PKC) and TGF-β1 (92).

**Figure 4:** Overview of the polyol pathway, adapted from Brownlee, 2001 (26). GSSG=Oxidized glutathione, GSH=Reduced glutathione.
The hexosamine pathway turns fructose-6 phosphate (created during glycolysis) into N-acetyl glucosamine, which modifies transcription factors via glycosylation (89) (fig. 5). For example the transcription factor Sp1 becomes glycosylated, and as a consequence, the expression of TGF-β and plasminogen activator inhibitor-1 (PAI-1) increase, both associated with diabetic complications. If the hexosamine pathway is inhibited there is no increasing TGF-β production in response to glucose (93). The hexosamine pathway has also been associated with oxidative stress. Glucose induced activity of the hexosamine pathway is prevented by overexpression of antioxidants, like manganese superoxide dismutase, MnSOD (94), which suggests that mitochondrially produced ROS have a stimulating effect on this pathway.

Fructose-6-P $\rightarrow$ GFAT $\rightarrow$ Glucosamine-6-P $\rightarrow$ N-acetyl glucosamine (GlcNAc) $\rightarrow$ Activation of transcription factor Sp1 $\rightarrow$ PAI-1 $\rightarrow$ TGF-β

**Figure 5:** Overview of the hexosamine pathway, adapted from Brownlee, 2001 (26). GFAT=Glutamine:fructose-6-phosphate amidotransferase

*Non-enzymatic glycation of proteins*

Glucose interacts with free amino groups in proteins and forms advanced glycation endproducts, AGEs, via non-enzymatic glycation. Hyperglycaemia causes an increase in the non-enzymatic glycation process. Formation of intracellular AGEs can lead to modifications of proteins inside the cells and
extracellular accumulation of AGEs can contribute to diabetic complications by crosslinking proteins in the ECM, for example collagen and laminin, thereby disturbing the signalling between the ECM and the cells (95). In murine diabetic nephropathy there have been observations of AGE accumulation in renal basement membranes (96). Extracellular AGEs interact with cell-surface receptors and trigger secretion of cytokines and growth factors (97) that contribute to the development of diabetic complications. The formation of AGEs also involves oxidation reactions (98) and stimulation of the AGE receptor can trigger ROS-production (99, 100), which in case of excess production of AGEs, will cause increased oxidative stress.

Reactive oxygen species
Hyperglycaemia-induced oxidative stress has been suggested as the unifying mechanism causing the cell damage seen in diabetic complications (26, 89). The regulation of a number of enzymes and signalling systems are influenced by the production of reactive oxygen species (ROS) (26, 94, 101). Hyperglycaemia also stimulates the synthesis of diacylglycerol (DAG), an activator of PKC, which is an intracellular signalling molecule affecting the expression of many other genes. For example PKC down-regulates eNOS and up-regulates PAI-1 (102, 103). The transcription factor NF-κB is up-regulated, which activates growth factors (TGF-β and VEGF) and inflammatory cytokines (89) (fig. 6). Both animal and in vitro studies have associated ROS with increased glomerular albumin permeability (104, 105) and antioxidant treatment has been shown to reduce glomerular hypertrophy, TGF-β1 expression and albuminuria in diabetic rat models (106, 107).

If the glycolytic enzyme GAPDH (glyceraldehyde-3 phosphate dehydrogenase) is inhibited, there is an accumulation of intermediate glycolysis products. In response to this, the polyol and hexosamine pathways are activated as well as PKC and the formation of AGEs. Overproduction of the antioxidant enzyme MnSOD or uncoupling protein-1 (UCP-1)* prevents the ROS-dependent inhibition of GAPDH and the

* Regulates the membrane potential in the mitochondria to control the electron transport chain.
following activation of PKC, the polyol and hexoxamine pathways and AGE formation (94, 108). Taken together this suggests that oxidative stress contributes to the development of DN.

**Figure 6:** During hyperglycaemia the formation of AGEs increase and also activation of PKC, leading to activation of growth factors and inflammatory cytokines resulting in increased risk of DN. Hyperglycaemia also increases ROS formation, which can inhibit GAPDH and cause further increase of the intermediate glycolysis products.

When glucose is processed by the mitochondrial respiratory chain (during the production of ATP) reactive oxygen molecules are produced in high amounts. During this process, electrons are transferred to intermediate electron carriers, such as NAD$^+$, and finally to oxygen, creating ROS (109). To protect the cells from the effects of oxygen radicals there are peroxidases, superoxide dismutases and different vitamins. There are two
INTRODUCTION

types of intracellular superoxide dismutases, the mitochondrial manganese superoxide dismutase (MnSOD) and the copper-zinc superoxide dismutase (CuZnSOD). In the mitochondria MnSOD catalyzes the reaction: 2 O$_2^-$ + 2 H$_2$O $\rightarrow$ O$_2$ + 2 H$_2$O$_2$. CuZnSOD on the other hand scavenges H$_2$O$_2$ in the cytoplasm. The enzyme MnSOD is encoded by nuclear DNA (the gene name is SOD2) and translocated into the mitochondrial matrix after translation. For optimal translocation, the signalling peptide guiding the MnSOD into the mitochondria is essential. A SNP, rs4880, has been identified in the targeting sequence resulting in a valine (Val) to alanine (Ala) exchange (Val16Ala) (110). An in vitro study has suggested that the Val-allele gives less efficient transport of MnSOD into the mitochondrial matrix (111), which can compromise the ability to neutralize superoxide radicals in the cell. Homozygous SOD2 knockout mice die shortly after birth suffering from metabolic acidosis, cardiomyopathy and lipid accumulation in the liver and skeletal muscles (112), while heterozygous SOD2 knockout mice show altered mitochondrial function compared to wild type mice (113, 114). This suggests that MnSOD is important for proper neutralization of ROS and could thereby have an effect on the risk of developing DN.

Glomerular composition and inflammation

Accumulation of ECM and mesangial expansion

Progression of DN depends on the accumulation and/or altered composition of ECM, which is controlled by a balance between synthesis and degradation of matrix components. When mesangial cells are cultured in a hyperglycaemic environment the production of ECM proteins, like laminin, fibrinectin and type IV collagen, increase (115) and when podocytes are cultured in high glucose, the balance between different ECM components is disturbed (116). In early diabetes, the glomerular hyperfiltration is also believed to induce hypertrophy and thickening of the glomerular basement membrane (117), which contributes to the development of DN.

Degradation of ECM is primarily performed by matrix metalloproteinases and the serine proteinase plasmin, which also activates matrix metalloproteinases (118). Plasminogen is turned into its active form, plasmin, by plasminogen activator (PA). PA is inhibited by plasminogen
activator inhibitor-1 (PAI-1), so PAI-1 indirectly inhibits the activation of plasmin (fig. 7). High glucose concentration leads to a reduction of PA and an increase of PAI-1 (119). Synthesis of ECM components has been shown to increase in response to glucose treatment in cultured mesangial and tubular epithelial cells (115, 120) (fig. 7), which can contribute to the accumulation of ECM and the expansion of the mesangium seen in DN.

![Figure 7: Hyperglycaemia reduces the amount of plasminogen activator (PA) and increases plasminogen activator inhibitor 1 (PAI-1), which also inhibits PA. PA is needed for the activation of plasmin, which degrades extracellular matrix (ECM). Thus, hyperglycaemia can lead to accumulation of ECM.](image)

The selectivity in the glomerular filtration depends on the pore size and the negative charge of the ECM and the glomerular basement membrane. The main component of the glomerular basement membrane is the negatively charged heparan sulphate proteoglycan, and both animal and human studies have shown that diabetes leads to decreased heparan sulphate content, loss of negative charges in the basement membrane and loss of charge selectivity in the glomerular filtration (121-123). It has been suggested that the defective metabolism of heparan sulphate proteoglycan causes albuminuria and other complications in diabetes (19). These changes in heparan sulphate proteoglycan content could not be found during early diabetes (124), thus it may be a factor of progression rather than initiation of DN.

**Inflammation associated genes in diabetes and its complications**

Many genes implicated in the development of T1D are involved in the maturation and function of the immune system. The human leukocyte
antigen (HLA), cytotoxic T-lymphocyte antigen-4 (CTLA4), the insulin gene† and PTPN22 (a suppressor of T-cell development and activation) (125) have all been linked to T1D. Also the migration of lymphocytes into the pancreas plays an important part in the process of autoimmune destruction of β-cells. This migration depends on adhesion molecules expressed on cell surfaces (126), for example the intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is involved in the migration and activation of lymphocytes (126-128) and both animal and human studies have addressed the importance of ICAM-1 in the development of T1D (129-133). The adhesion and de-adhesion of inflammatory cells, and also homing to target tissues, can be influenced by genetic polymorphisms in the ICAM1 gene. The E469K polymorphism (rs5498) and R241G (rs1799969) have been studied in different populations and the E469K have been associated with T1D in a Japanese population (134), but this could not be repeated in a Danish population (135) or in Caucasian families from Europe and USA (136). In European and American families the R241 allele was transmitted significantly less often to T1D-affected offspring, suggesting that this is a protective allele (136).

ICAM-1 can also be involved in the development of DN (137) and other diabetic long-term complications, like atherosclerosis and cardiovascular disease (138). Infiltration of mononuclear cells into the glomeruli, which can be mediated by ICAM-1 (137), has been observed in patients with diabetes (139, 140). Cyclic stretching of mesangial cells in culture, simulating intraglomerular hypertension, up-regulates ICAM-1 (141) and it has been suggested that glomerular hyperfiltration induces expression of this adhesion molecule (137). A study including T2D patients have correlated decreased renal function with increased levels of ICAM-1, even stronger correlation was found with vascular cell adhesion molecule-1 (VCAM-1) (142). Animal studies have shown that ICAM-1 deficient diabetic mice have lower urinary albumin excretion, less glomerular hypertrophy, and less mesangial matrix expansion than mice with normal expression (143, 144), suggesting that ICAM-1 is involved in the pathogenesis of DN. To my knowledge there are no previous studies of association between polymorphisms in the ICAM1 gene and DN.

† Reduced expression of insulin in the thymus can lead to mature T-cells that recognise insulin as non-self.
Inflammatory markers, such as C-reactive protein (CRP) and interleukine-6 (IL-6), have been associated with T1D and with DN (145). Increased levels of CRP and tumour necrosis factor-α were found also in T2D patients and the levels of these markers were correlated to urinary albumin excretion (146). In addition there are studies showing that an activated complement system‡ can be involved in the pathogenesis of DN (147-150). The main mission of the complement system is to alert the immune system of intruding pathogens and help in their destruction, but there is also evidence of complement activation after oxidative stress (151). Thus, inflammatory mechanisms seem to be involved both in the development of diabetes and its complications.

Non-Genetic Factors

Metabolic control and genetic background can influence DN risk, but also environmental and lifestyle related factors are important. External stimuli like diet and smoking are suggested to contribute and it is speculated that intrauterine growth retardation and low birth weight results in a reduced number of nephrons leading to a high workload for each nephron and thereby increases the risk of DN (152, 153). Nutritional substances play a role in the development of diabetic complications. Apart from influencing metabolic control, it is also possible to affect cardiovascular risk factors like hyperlipidemia, and to reduce the strain on the diabetic kidneys by choosing the right diet.

Dietary intake, reduce or increase?

Proteins

Already in 1932 Shannon et al. found that changing the diet of dogs, from carbohydrate to protein based, increased the renal blood flow and GFR (154). Pullman et al. showed in 1954 that this also happened in man (155). High intake of protein has been suggested to contribute to the development and progression of kidney disease and it is known to increase renal

‡ A biochemical cascade of the immune system that recruits inflammatory cells, tags the pathogens for recognition, or destroys the targets through disruption of the plasma membrane.
workload and GFR (156). In normoalbuminuric T1D patients, a diet containing low amounts of protein can normalize glomerular hyperfiltration (157). To lower the dietary intake of protein has been used as treatment for chronic kidney disease for a long time, but the effectiveness of this treatment is not totally elucidated (158, 159). Today the recommended daily protein intake for T1D patients, without albuminuria, is 10-20% of the total caloric intake (if total intake is 2500 kcalories, 10-20% represents 56-111g protein). Patients with DN are recommended to limit their protein intake to 0.6-0.8g/kg bodyweight (160) (if a person weighs 75kg this corresponds to 45-80g protein).

It is possible that dietary proteins from different sources have different renal effects. Proteins of animal origin are proposed to cause a heavier burden on renal haemodynamics than vegetable protein (156, 161) and a substitution of red meat with white meat (chicken and fish) can reduce GFR in hyperfiltrating patients (162) and lower albumin excretion rate in macroalbuminuric T2D patients (163). Thus, not only the amount of protein eaten, but also the protein source can be important in planning dietary changes for T1D patients.

**Fatty acids and vitamins**

Besides protein, normal diet contains other substances that can affect the kidneys, for example vitamins and different fatty acids. There is evidence that a diet rich in fish reduces levels of triglycerides, low density lipoproteins and cholesterol (164). Fish consumption is also inversely related to cardiovascular mortality (165, 166). Fish oil, containing polyunsaturated fatty acids, mainly from the \( \omega_3 \)-class, has been found to reduce progression of IgA nephropathy (167, 168). In an animal model of this disease, fish oil also decreased glomerular damage and cell proliferation (169). In diabetic nephropathy, however, it is not clear that fish oil has beneficial effects (170, 171). Antioxidants, like vitamins E and C, have been found to protect from DN in animal studies (107, 172-175), but studies with humans have not been conclusive (176-178). There is also evidence of increased mortality associated with excess vitamin E supplementation (179), which speaks against the use of vitamin E as preventive medication for DN.
Cigarette smoking

Many studies have shown that smoking is significantly associated with the prevalence and progression of DN and cigarette smoking is accepted as a risk factor for DN. However, not all smokers are affected (180-185), thus implicating that combinations of different factors are needed.

Smoking and renal haemodynamics

Smoking T1D patients, with normoalbuminuria, have higher blood pressures than non-smoking (186), and smoking T1D patients also have a higher baseline GFR and a more rapid GFR decrease than non-smoking patients (187). A study on the immediate effects of smoking showed that when smoking a cigarette, the blood pressure raises, the pulse rate goes up and the plasma levels of aldosterone and cortisol increase (188). Taken together, this can be part of the explanation for the increased risk of diabetic kidney disease among smokers, and it may be reinforced by anomalies in the renal haemodynamic control systems.

Smoking and oxidative stress

Since cigarette smoke contains oxygen radicals (189) and increases the demand for antioxidants (190), oxidative stress is a possible link between smoking and DN. The hyperglycaemia-induced ROS-production in diabetes can be pronounced by the oxidative stress caused by smoking and lead to additional strain on the defence systems. Hyperglycaemia is necessary but not sufficient for the development of DN and the individual response to environmental risk factors, such as smoking and diet, can be genetically determined. It is highly likely that both genetic and non-genetic factors interact in the development of this complication.
Objectives

The general objective of this thesis was to evaluate the influence of a number of genetic and lifestyle related factors on the risk of diabetic nephropathy in T1D patients.

More specifically, the research questions were:

- Do genetic polymorphisms in the blood pressure regulating *NOS3* and renin-angiotensin-aldosterone system genes, influence the risk of diabetic nephropathy in T1D patients?
- Does a functional polymorphism in the *SOD2* gene influence the risk of diabetic nephropathy in T1D patients?
- Do genetic polymorphisms in the *ICAM1* gene influence the risk of having T1D and DN?
- Does the combination of smoking and genetic factors affect the risk of diabetic nephropathy?
- Does normal diet, especially protein intake, influence the risk of having microalbuminuria in young Swedish T1D patients?
Subjects and Methods

This thesis is based on data from three different sample sets. The first consists of young T1D patients from Sweden who participated in the dietary study. The second group consists of Swedish participants having longer duration of T1D, with or without DN. The third group is a large sample set of Finnish T1D patients, with or without DN.

Participants in the dietary study (paper I)

The study of dietary influence on microalbuminuria in young T1D patients was done in a population based nested case-control manner. All T1D cases registered in the national diabetes registry between the 1st of July 1977 and the 31st of December 1987 were invited. Out of the 3858 invited patients, 1150 accepted to participate. They answered a semiquantitative food frequency questionnaire and sent urine samples for analysis of microalbuminuria. A total of 75 participants were found to have microalbuminuria, defined as albumin excretion rate $>15\mu g/min$ in at least two over night samples. From the remaining participants, 225 duration-matched controls were chosen (fig. 8). In this setting the power was 80% to detect a doubling of the risk (SPSS SamplePower 2.0).

Figure 8: Nested case-control study flowchart.
SUBJECTS AND METHODS

The minimum diabetes duration was five years and the mean duration was approximately 11 years (table 1, paper I). Clinical information, for example current HbA1c, blood pressure, weight and height, was obtained from medical records.

The food frequency questionnaire was developed and evaluated by the Swedish National Food Administration (191). There was also a computer program that converted the answers into amount of nutrients. The food frequency questionnaire contained pictures of standard size dishes to help the participants estimate the amount of food eaten (fig. 9). The questions concerned how often the participants had consumed different kinds of dishes, sandwiches and drinks during the last 12 months, and also how the food was prepared. Example of questions:

How often do you eat fish (times per day, week, month)?
How was it prepared (boiled, fried, smoked, baked, with breading)?

Figure 9: Four standard size dishes used for estimation of the amount of food consumed.
Participants in the genetic studies (paper II-V)

Since DN often develops at 15-20 years of diabetes duration, participants in the genetic studies had longer duration than the participants in study I. Patients included as controls had at least 20 years of diabetes duration and were free from albuminuria, AER <20μg/min. To avoid misclassification of incipient DN cases as controls, an important inclusion criterion for the controls was that they had no antihypertensive treatment, which might disguise a possible microalbuminuria.

The cases had either incipient or overt DN. Incipient cases had microalbuminuria, defined as AER between 20 and 200μg/min in at least two consecutive overnight samples. Overt cases had AER >200μg/min, or received renal replacement therapy (dialysis or kidney transplantation). There were no limitations for the cases regarding antihypertensive treatment, but the duration to DN onset was at least 5 years. In Sweden we mainly included patients from two medical clinics, Karolinska Hospital (n=165) and Umeå University Hospital (n=153) (paper II-V). In paper III, additional patients from the Karolinska Hospital were included (n=114) and also 187 non-diabetic healthy controls, without T1D, were recruited. The mean age of the healthy controls was 48 years and none had any family history of diabetes (type 1 or type 2), thus they were not likely to develop T1D. In the later studies (paper IV-V) T1D patients undergoing renal replacement therapy in other Swedish clinics were added to the participants from study II (n=29). The Finnish sample set included patients from all over Finland and was a part of the FinnDiane study (n=1163) (paper IV and V). Table 2 shows an overview of the number of participants in each study. Clinical data are given in each paper.

Table 2: Numbers of cases and controls in each study.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Type of study</th>
<th>Cases overt DN</th>
<th>Cases incipient DN</th>
<th>Controls no DN</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Nested population based case-control</td>
<td>Swedish n=75</td>
<td>Swedish n=225</td>
<td></td>
</tr>
<tr>
<td>II.</td>
<td>Case-control</td>
<td>Swedish n=48</td>
<td>Swedish n=73</td>
<td>Swedish n=197</td>
</tr>
<tr>
<td>III.</td>
<td>Case-control</td>
<td>Swedish n=67</td>
<td>Swedish n=129</td>
<td>Swedish n=236</td>
</tr>
<tr>
<td>IV-V.</td>
<td>Case-control</td>
<td>Swedish n=78</td>
<td>Swedish n=72</td>
<td>Swedish n=197</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>Finnish n=541</td>
<td>Finnish n=264</td>
<td>Finnish n=358</td>
</tr>
</tbody>
</table>

*In study III also 187 non-diabetic controls were included.
Blood pressure and HbA1c values were either measured at inclusion or taken from the patient's medical records, where these values are entered at least once a year. The participants answered a questionnaire including questions on smoking habits. To be regarded as a smoker, at least one cigarette per day for at least one year had to be consumed. Current smoking or ex-smoking were considered as “ever smoking”, and patients answering that they were not current smokers or ex-smokers were considered as never having smoked. If the question on smoking was not answered, the participant was excluded from the calculations that regarded smoking.

Genetic markers

The genetic markers used in studies II-V were mostly single nucleotide polymorphisms (SNP), also a few microsatellite markers and insertion/deletion polymorphisms were used. SNPs are very common sequence variations occurring when a nucleotide (A, C, T or G) in a specific genomic site is different in two different individuals, or in paired chromosomes in the same individual. The SNPs can be located in the coding or non-coding region of genes, or between genes. In the coding region of a gene, SNPs can lead to changes in the amino acid sequence that can affect the function of the protein. In non-coding regions transcription, translation and gene splicing can be affected.

Most often a SNP has two alleles while a microsatellite marker has 2-10 or even more alleles. Microsatellite markers are less common than SNPs but they are usually very informative, due to the many different alleles, and they are the most common markers used in genome-wide scans and linkage analyses. In study II we used two microsatellite markers previously used to study hypertension DN, one located adjacent to the angiotensinogen gene and the other close to the AGTR1 gene. The angiotensinogen marker has been linked to hypertension in some studies (192) but not all (193) and could qualify as an interesting marker also for DN. The marker located near the AGTR1 has been linked to DN in T1D siblings discordant for DN (194).

Insertion/deletion polymorphisms are characterised by insertion or deletion of DNA segments, from one base to several hundred, and they can cause a lot of changes in affected genes.
Statistical methods

For the statistical analyses SPSS for Windows was used. Power estimations were made using either SPSS Sample Power for Windows or the Genetic Power Calculator, developed by Purcell et al. (195). To determine linkage disequilibrium (D’ and r²) the Haploview 3.2 software was used (196). Conventional statistical methods (Pearson Chi² and Fishers exact test) were used to compare differences between groups.

To assess independence of different factors, logistic regression analysis was used. Additive interaction was studied by stratification and calculation of the Relative Excess Risk due to Interaction (RERI), as suggested by Rothman (197). There is no sign of interaction (departure from additivity) if the RERI is 0. The following formula is used:

\[
RERI = 1 + OR_{A+B} - OR_{A+B} - OR_{A-B}
\]

In studies I and III-V P-values less than 0.05 was considered statistically significant. In paper II the null-hypothesis was not restricted to a few polymorphisms in one gene, but rather polymorphisms and markers in a set of genes. So to make some correction for multiple testing, P-values less than 0.01 were considered statistically significant.
RESULTS AND DISCUSSION

Results and Discussion

It is known that diabetic nephropathy is a disease with complex pathogenesis that depends on genetic predisposition as well as factors associated with environment and lifestyle. In this thesis the risk of DN has been addressed from both perspectives. Some possible interactions between these different mechanisms have also been explored.

Results and Discussion of Each Paper

Diet and DN in young T1D patients (paper I)

In paper I the dietary habits of young T1D patients were semi-quantitatively measured using a food frequency questionnaire. Microalbuminuria, a predictor for overt diabetic kidney disease (7, 18, 198, 199), was observed in 75 participants. Three duration matched controls were chosen per case (n=225). Clinical characteristics are shown in table 1, paper I. Cases were older (P=0.02), had a later onset of diabetes and they had higher blood pressure than the controls (P<0.01).

When comparing mean eating habits of cases and controls there were no differences in intake of energy, fat, protein or carbohydrates (table 1, paper I). After dividing protein (table 2, paper I) and fat (table 3, paper I) according to origin (fish, meat, milk or vegetable), mean intake of fish and milk protein tended to be higher among control subjects, but no differences were observed regarding mean intake of fat from different sources.

To evaluate how a high intake (≥75th percentile) of protein or fat from different sources affected the risk of having microalbuminuria, odds ratios and 95% confidence intervals (CI) were calculated. This showed that a high intake of fish protein was a significant protective factor, OR=0.49 (95% CI=0.25-0.97), the same was found regarding fish fat. A high milk protein intake tended to be a protective factor, but did not reach significance, 0.55 (0.28-1.07), and for milk fat the OR was 0.76 and 95% CI=0.41-1.43. A logistic regression analysis (table 4, paper I) showed that an intake of ≥75th percentile of fish protein was a protective factor independently of age, sex, region of origin, duration of diabetes, HbA1c, mean arterial pressure, BMI, energy intake, smoking and high fish fat intake. A high intake of fish
protein corresponded to a mean intake of 53 g fish per day (9.3 g fish protein/day), which means that if a normal size serving is 125-175 g fish, the high consumers had fish on the average every third day. It is recommended that diabetic patients keep a moderate protein intake to reduce the workload and risk of DN (160). In this study of young T1D patients the recorded mean protein intake was relatively low, as recommended for diabetic patients, and we could not find any association between protein intake and increased risk of microalbuminuria.

It is unlikely that the data had a disease dependent bias, since microalbuminuria was determined after the participants answered the questionnaires, and if the cases were aware of their microalbuminuria, it is unlikely that they would give a biased response regarding fish consumption. Not finding associations between microalbuminuria and dietary intake must be cautiously interpreted. We cannot exclude nondifferential misclassification of exposure (same misclassification in cases and controls) which would dilute the ORs.

In this study it seemed like the fish protein had a beneficial effect on risk of microalbuminuria, but it is not clear whether the protective effect is provided by the fish protein or the fish fat. It is possible that protein from fish has a different composition of amino acids than meat protein and thus can have different effects in the body. An animal study by Yahia et al. showed decreased superoxide dismutase contents in different tissues and beneficial effects on blood pressure and plasma cholesterol in spontaneously hypertensive rats fed fish protein for two months compared to rats fed casein (200), suggesting that fish protein decreases both blood pressure and the need for antioxidants.

Previous studies have shown that fish oil have effects like lowering blood pressure and triglyceride levels (201-204), but also that it reduces albuminuria and has positive effects on kidney function in patients with different kinds of nephropathy (170). Even though our study shows that fish protein is associated with protection against microalbuminuria also when adjusting for fish fat, we cannot conclude that fish fat does not have a beneficial effect.

In conclusion the study shows that an intake of fish protein in the highest quartile had a protective effect and lowered the risk of having microalbuminuria. A normal consumption of protein, even in the highest
RESULTS AND DISCUSSION

quartile, did not increase the risk of having microalbuminuria but we cannot exclude that a very high intake of protein has a detrimental effect, since this study was conducted to see the effects of a normal diet.

The renin-angiotensin-aldosterone system and DN (paper II)

Study II investigated seven genetic markers in the renin-angiotensin-aldosterone system. The included genes (table 3) have been implicated as candidate genes for DN and most of the markers have been studied in association with DN with varying results. We wanted to see if these polymorphisms affected the risk of DN among Swedish T1D patients.

Table 3: Genes and markers included in study II.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Insertion/Deletion</td>
</tr>
<tr>
<td>AGT</td>
<td>M235T, A-20C, microsatellite marker</td>
</tr>
<tr>
<td>AGTR1</td>
<td>A1166C, microsatellite marker</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>C-344T</td>
</tr>
</tbody>
</table>

The participants in study II were Swedish Caucasian T1D patients. There were 48 patients with overt DN, 73 with incipient DN and 197 controls with diabetes but without DN. Since we tested seven markers in four different genes there can be a risk of false positive results and to avoid this we chose to consider a P-value of <0.01 as significant instead of the more common <0.05. The power of the study was 80% to detect approximately 2.5 times risk increase.

Two markers in different genes indicated association with DN, the microsatellite marker close to the AGT gene and the A1166C polymorphism in the AGTR1 gene. When performing an overall chi$^2$-test, the A1166C marker indicated differences between controls and cases with overt DN, P=0.03 (table 2, paper II). From the genotype frequencies it seems that the AA-genotype is more common among the overt DN cases than among the controls. When analysing the polymorphism further, a recessive model of the A-allele was chosen. Comparing overt DN cases with controls in a logistic regression analysis including the variables: age, duration of diabetes, sex, ever smoking and HbA1c, showed that the effect...
RESULTS AND DISCUSSION

of the gene was statistically significant when taking these variables into account. Having AA-genotype significantly increased the risk of overt DN about three times, adjusted OR=3.04 (99% CI=1.02-9.06), P=0.009. In the group of patients that were smokers or ex-smokers and had AA-genotype the risk was even more pronounced, compared to not having any of the risk factors, OR=4.84 (99% C.I.=1.02-22.97), P=0.009. The RERI was calculated to 2.93 (table 5, paper II), suggesting that there is a synergistic interaction between the AGTR1 gene and smoking. For patients with both risk factors this interaction results in higher risk than expected.

Two alleles (the 125 and 127 bp alleles), of the AGT microsatellite marker, were more common in controls than in DN cases (table 4, paper II), which suggested a protective effect of these alleles. In a crude chi\(^2\) analysis carrying either the 125 or the 127 bp allele was significantly associated with protection against having overt DN, OR=0.30 (99% CI=0.10-0.94) P=0.007. When adjusting for other possibly associated factors (age, duration of diabetes, sex, ever smoking and HbA1c), the alleles were no longer significantly associated with DN.

None of the other markers were associated with DN in this study. It is, however, possible that a larger study would have been able to detect association and that the negative findings are due to the rather small number of patients.

**ICAM1 is associated with T1D but not with DN (paper III)**

The aim of study III was to investigate how genetic polymorphisms in the *ICAM1* gene influence the risk of T1D and DN. The adhesion molecule ICAM-1 is important for the migration and activation of lymphocytes, which is essential for the autoimmune destruction of \(\beta\)-cells. There is also increased expression of ICAM-1 in the kidneys of diabetic rats (137, 205), suggesting that this molecule can affect the risk of DN.

The participants in study III were 432 T1D patients and 187 non-diabetic healthy controls. Among the T1D patients 196 had AER >20 \(\mu\)g/min and were considered as having DN. Five SNPs in the *ICAM1* gene, with allele frequencies higher than 1%, were included in the analyses. Genotype frequencies of two of the studied SNPs were significantly different in T1D patients and non-diabetic control subjects. The
polymorphisms were the intronic rs281432 C/G polymorphism and rs5498 A/G, located in exon 6 and causing a lysine to glutamic acid exchange. In dominant models of the common alleles, both SNPs were significantly associated with T1D, rs281432 OR=1.64 (95% CI=1.14-2.38), P=0.008, and rs5498 OR=2.46 (95% CI=1.59-3.80), P=0.001 (table 4, paper III). The linkage disequilibrium was moderately high between these two SNPs. Haplotype analyses were performed and the haplotype consisting of the two common alleles (rs281432 C and rs5498 A) was significantly more common in T1D patients than in non-diabetic controls, P=0.035 (table 6, paper III).

There were also gradual increases in the frequencies of the rs281432 C- and rs5498 A-alleles from non-diabetic controls (C=40.1% and A=51.1%) to T1D patients without DN (C=44.5% and A=54.7%) and to T1D patients with DN (C=47.2% and A=57.4%), but the increase was not statistically significant. The rs5498 causes an amino acid exchange, E469K (lysine to glutamic acid), in exon 6 and this site may affect mRNA splicing and apoptosis. A study by Iwao et al. suggests that A/A cells have a lower sensitivity to apoptosis than G/G cells, which could result in higher survival of auto-reactive immune cells and increased risk of autoimmune diseases like T1D (206).

**NOS3, smoking and DN (paper IV)**

In study IV, we tested the hypothesis that two genetic polymorphisms in the NOS3 gene were associated with increased risk of having DN and also that smoking may modify the genetic risk. The two polymorphisms were chosen because they have been associated with hypertension (78) and cardiovascular risk in previous studies (79), and they have also been studied in relation to both diabetic (tables 1a and 1b) and non-diabetic renal diseases (76, 80, 81), with varying results.

We had the opportunity to investigate Swedish and Finnish T1D patients. See the Subjects and Methods section and paper IV for details on the inclusion criteria. The genotype and allele frequencies of the NOS3-polymorphisms did not differ between the Finnish and the Swedish sample sets and they were in Hardy Weinberg equilibrium separately and together,
so despite differences in the clinical characteristics (table 1, paper IV) they were considered similar enough to be combined.

Linkage disequilibrium between the two polymorphisms were $D' = 0.92$, but the alleles were not correlated, $r^2 = 0.07$. In $3 \times 2$ or $2 \times 2$ contingency tables, no associations were found between the genotype or allele frequencies and DN (table 2, paper IV). We chose to analyze homozygosity for the common alleles vs. heterozygosity or homozygosity for the uncommon alleles in univariate analyses, but no associations with DN were found. Multivariate logistic regression analyses were performed to reduce the variability of the outcome caused by other associated factors and to look at independence from these factors. The variables included were; age at onset, duration of diabetes, HbA1c, smoking, sex, systolic and diastolic blood pressure. Significant and independent association was found with homozygosity for the Glu-allele of the Glu298Asp polymorphism, OR=1.46 (95% CI=1.12-1.91), $P=0.005$ (table 3, paper IV), but not with the NOS4ab polymorphism OR=1.00 (95% CI=0.75-1.33). The other included variables contributed significantly to all models.

Smoking is clearly a contributing factor in the development of DN. In this study the risk of having DN was doubled by ever smoking, OR=2.00 (95% CI=1.60-2.50), $P<0.001$ (ever smoking included both current smoking and ex-smoking). Smoking is suggested to impair NO-dependent vasodilation (207) and affect the regulation of coronary artery tone (208), which may result in disturbed regulation of the intraglomerular pressure. Since not all smoking T1D patients develop DN, the risk associated with smoking may be enhanced or reduced by genetic factors, so the effect of smoking can be modified by polymorphisms in NOS3.

To analyse the possible interaction between smoking and genotype, the patients were stratified according to smoking status. In the combined sample set, including both Finnish and Swedish T1D patients, no interaction was found with either the NOS4ab or the Glu298Asp (table 4) polymorphisms.
RESULTS AND DISCUSSION

Table 4: Effect modification of NOS3 Glu/Glu and smoking in Finnish and Swedish T1D patients.

<table>
<thead>
<tr>
<th>NOS3 Glu/Glu</th>
<th>Smoking</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>1.00 (Ref.)</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>&lt;0.001</td>
<td>1.94 (1.39-2.71)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>0.096</td>
<td>1.32 (0.95-1.84)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>&lt;0.001</td>
<td>2.41 (1.71-3.40)</td>
</tr>
</tbody>
</table>

No DN Controls vs. DN cases. Values are adjusted for age at diabetes onset, diabetes duration, HbA1c and sex. RERI= 0.15, no departure from additivity.

There were tendencies for increased risk associated with the Glu/Glu genotype in both the ever smoking and never smoking groups, but they did not reach statistical significance. When analysing the Finnish and Swedish participants separately, there was a significantly increased risk of DN associated with the NOS4a-allele in the Swedish ever smoking group but not the Finnish. These results have to be interpreted carefully since there may be statistical fluctuations because of the rather small number of Swedish patients after stratification on smoking status. However, in the ever smoking group there are 148 patients in total, and this is about the same number as some previous studies have used (table 1b, introduction). There is also a possibility of differential misclassification of smoking in the two countries that may affect the result of the interaction studies.

SOD2, smoking and DN (paper V)

The biological background for study V was that an impaired transport of MnSOD into the mitochondria can result in increased oxidative stress. In a hyperglycaemic environment, suggested to be sensitive to changes in antioxidant status, this may increase the risk for damage. A genetic polymorphism known to affect both translocation of MnSOD into the mitochondria and mRNA stability (111, 209), was investigated. The hypothesis was that the allele impairing translocation could increase the risk of DN.

This polymorphism was investigated in Swedish and Finnish T1D patients, for details on the inclusion criteria see the Subjects and Methods section and paper V. The genotype and allele frequencies of the SOD2 Val/Ala-polymorphism did not differ between the Finnish and the Swedish
RESULTS AND DISCUSSION

sample sets and they were in Hardy Weinberg equilibrium separately and together, so they were combined.

In a logistic regression analysis including age at onset, duration of diabetes, HbA1c, smoking and sex, the Val/Val-genotype was associated with slightly increased risk of DN OR=1.32 (95% CI=1.00-1.74), P=0.049. This suggests that the neutralization of oxygen radicals in the mitochondria, by MnSOD, is important in lowering the risk of DN.

Smoking increased the risk of DN OR=2.00 (95% CI=1.60-2.50), P<0.001, and since smoking is known also to increase the amount of oxygen radicals, the combined effect of smoking and the SOD2 Val/Val genotype was investigated using a stratification of genotype and smoking status in a logistic regression adjusting for age at diabetes onset, duration of diabetes, HbA1c and sex, and. In the combined Finnish and Swedish sample set, there was no further increase in risk for ever smoking patients having the Val/Val-genotype (table 3, paper V). Among the Swedish patients, however, there was an indication of synergistic interaction. Compared to the low risk group (never smoking and not being Val-homozygous), the high risk group (ever smoking and having Val/Val genotype) had OR=2.95 (95% CI=1.26-6.91) while smokers without the Val/Val genotype had OR=0.94 (0.53-1.68). Only Val/Val genotype, but never smoking gave a non-significant OR of 1.26 (0.51-3.13), resulting in a departure from additivity of 1.75 (fig. 10). The risk of having DN was increased when having both associated factors and the increase was higher than would be expected if just adding the risk from smoking with the risk from the genotype.

MnSOD is an important enzyme in the process of neutralizing reactive oxygen species and if it is not translocated properly into the mitochondria the effect will be an excess of ROS that can result in damage on cells and tissues, especially during the extra stress caused by hyperglycaemia and smoking.
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Figure 10: Graph illustrating the joint effect of the SOD2 Val/Val-genotype and smoking in the Swedish sample set. 1= risk set to 1 when carrying none of the studied factors, 2=ever smoking, not carrying risk genotype, 3=never smoking, carrying risk genotype, 4=smoking and carrying risk genotype, which also gives the excess risk due to interaction.

Gene-gene interaction

There are also possible gene-gene-interactions involved in disease development. The effect of one gene can be modified by another gene. Superoxide radicals are suggested to play a role in the endothelial dysfunction, seen in diabetes, by disturbing the regulation of eNOS (210). O$_2^-$ and NO forms a strong oxidizing agent, ONOO$^-$ (peroxynitrite), thus inactivating NO as a vasodilator (211, 212). Accumulation of peroxynitrite has been observed in diabetic kidneys (213). Additionally, oxidative radicals in serum from smokers have been found to upregulate eNOS expression but impair eNOS activity in vitro (214, 215). When studying the joint effect of the NOS3 Glu/Glu- and SOD2 Val/Val-genotypes in the combined Swedish and Finnish sample set there was no synergistic effect, RERI=-0.09, table 5 and no effect modifications were found in the Finnish and Swedish sample sets separately either.
Table 5: Gene-gene interaction $SOD2$ and $NOS3$-polymorphisms

<table>
<thead>
<tr>
<th>$SOD2$</th>
<th>$NOS3$</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val/Val</td>
<td>Glu/Glu</td>
<td>−−</td>
<td>1.00 (Ref.)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>0.080</td>
<td>1.41 (0.96-2.08)</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>0.042</td>
<td>1.33 (1.01-1.74)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.014</td>
<td>1.65 (1.11-2.46)</td>
</tr>
</tbody>
</table>

No DN controls vs. DN cases. Values are adjusted for age at diabetes onset, diabetes duration, HbA1c, smoking and sex. RERI=-0.09, no departure from additivity.

General Discussion

Summaries of the main aims, study designs and conclusions of all included studies are shown in table 6.

Methodological aspects

Differences and similarities in the sample sets

In studies IV and V we had the opportunity to collaborate with the FinnDiane group in Helsinki, Finland. The FinnDiane study is a Finnish nationwide multicenter study, aiming to identify risk factors for DN in T1D patients (216). One reason for including both Swedish and Finnish subjects was to increase the power of the analyses, in case the populations were to be regarded as similar in clinical and genetic aspects. Another reason was that this provided a possibility to test our hypotheses in two different populations. A risk marker that can be found in different populations would strengthen an association, whereas differential results could point at potential differences in modifying factors.

The inclusion criteria for the cases and controls were the same in Finland and Sweden and the frequencies of the genetic polymorphisms investigated were very similar in the Finnish and Swedish T1D populations, so for these studies the two sample sets were combined to achieve a gain in power. There may, however, be reasons for not combining sample sets from different countries. For example there can be differences in susceptibility, caused by variations in environmental factors or in background genes.
Table 6: Summary of papers included in the thesis.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Research question</th>
<th>Study design</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>How does normal diet, particularly protein intake, influence the risk of microalbuminuria in young Swedish T1D patients?</td>
<td>Nested population based case-control cases n=75, controls n=225</td>
<td>High amount of dietary protein did not increase the risk of microalbuminuria. High amount of fish protein (9.3 g fish protein per day, or more) was a protective factor.</td>
</tr>
<tr>
<td>II.</td>
<td>Do genetic polymorphisms in the RAAS influence the risk of diabetic nephropathy in Swedish T1D patients?</td>
<td>Case-control cases n=121, controls n=197</td>
<td>AA-genotype of the A1166C polymorphism in the AGTR1 gene was associated with overt DN. Smoking and AA-genotype had a synergistic effect, further increasing the risk.</td>
</tr>
<tr>
<td>III.</td>
<td>Do genetic polymorphisms in the ICAM-1 gene influence the risk of T1D and diabetic nephropathy in Sweden?</td>
<td>Case-control T1D cases n=432 (196 with DN), controls n=187</td>
<td>Two polymorphisms in the ICAM1 gene were significantly associated with T1D, but not with DN.</td>
</tr>
<tr>
<td>IV.</td>
<td>Do genetic polymorphisms in the NOS3 gene influence the risk of diabetic nephropathy in Swedish and Finnish T1D patients?</td>
<td>Case-control cases n=955, controls n=555</td>
<td>The Glu/Glu-genotype of the NOS3 Glu298Asp polymorphism increased the risk to develop DN, when taking other associated factors into account.</td>
</tr>
<tr>
<td>V.</td>
<td>Does the Val16Ala polymorphism in the SOD2 gene influence the risk of diabetic nephropathy in Swedish and Finnish T1D patients?</td>
<td>Case-control cases n=955, controls n=555</td>
<td>Homozygosity for the Val-allele of the SOD2 Val16Ala polymorphism was associated with increased risk of DN, when taking other associated factors into account.</td>
</tr>
</tbody>
</table>
Most of the clinical measurements were comparable, like the AER and blood pressure values, but when comparing HbA1c values we had to keep in mind the variation in normal range between Sweden and Finland. The upper normal limit in Sweden is 5.2% and in Finland it is 6.0%. We calculated new values based on percent of upper normal limit for each country. The upper normal limit was set to 100% and an HbA1c value of 7.0 equals 116.7% of reference in the Finnish sample set and 134.6% of reference in the Swedish sample set. This transformation makes the values comparable. The questions on smoking were put to the participants in questionnaires and the participants answered in writing. The questions were almost identical in the Finnish and Swedish questionnaires. Examples:

**Finnish example:**
- Do you smoke? Y/N
- If yes, when did you start smoking? Year:
- If yes, how many cigarettes/cigars do you smoke per day, on the average?

**Swedish example:**
- Do you smoke? Y/N
- If yes, how long have you been smoking? Years:
- If yes, how many cigarettes do you smoke per day, on the average?

Despite very similar questions and the use of questionnaires, there may be differential misclassification of smoking in Finland and Sweden. In Sweden 51.1% of the controls answered that they never smoked, but 61.6% of the controls in Finland never smoked. The difference between Swedish and Finnish controls was statistically significant, $P=0.03$. When comparing the cases, there was no difference between the countries in number of ever smokers or never smokers, $P=0.90$.

**Power**

Since development of DN depends on many different genes and environmental factors, each gene contributes with only one small piece of the puzzle and to find a gene with strong association to DN would be a rare event. In a meta-analysis by Fujisawa et al. (52), including almost five thousand patients from different studies, the thoroughly investigated insertion/deletion polymorphism in the ACE gene was associated with DN and the summary odds ratio was about 1.3. This means that very large
RESULTS AND DISCUSSION

studies are needed to detect such modest risk increases and it also illustrates the importance of avoiding publication bias so meta-analyses can be performed. When conducting case-control studies to find factors associated with multifactorial diseases like DN, power is one important aspect to consider. The study has to include enough participants to be able to find the association of interest and at the same time the number of participants is a question of access, time and money.

Another important consideration would be the accurate categorization of cases and controls, because if they are not correctly categorized the power will be reduced. In the genetic studies included in this thesis, the controls had at least 20 years of diabetes duration (median 28 years), without microalbuminuria and without any antihypertensive medications. Since the peak incidence is around 15-20 years of diabetes duration (1, 4, 27), it is reasonable to believe that patients who have not developed DN after 20 years have less genetic predisposition than patients developing DN early during diabetes. There is, however, a possibility that some of the controls may develop DN later on. This will lower the power of the study and make it more difficult to find genetic associations that really are there, increasing the risk of false negative results.

Participants in any stage of DN (microalbuminuria, macroalbuminuria or ESRD) were considered as cases. Even if microalbuminuria is a good predictor of progression to macroalbuminuria and ESRD, patients with microalbuminuria have a chance of reverting to normal albumin excretion values, especially if the metabolic control is improved (217-220), which may lower the power of the studies. We can also speculate that patients with long duration before developing DN may not be genetically predisposed. In study IV this issue was addressed by redoing the association calculations excluding patients with >30 years of diabetes duration before onset of DN. This did not change the results of the study.

Adjusting for confounders
Logistic regression can be used to build a model that explains a binary outcome. In epidemiology it is used to identify the combination of factors that explains the majority of disease risk. Logistic regression can also be used to adjust for confounders, to study the independence of contributing factors. Due to the complex aetiology of DN this method was chosen in
RESULTS AND DISCUSSION

our studies mainly to adjust for confounders. In our genetic studies the effect of the genes became significant after adjustment for other associated factors. When including the associated factors, the variability of the outcome depending on these factors, was reduced, and the effect of the studied factor could be detected. This means that the effect of the gene was no longer concealed by the shakiness caused by the other factors.

To determine which factors should be included, results from the present and previous studies must be considered. There are some factors that have been associated with DN in many studies, like HbA1c and smoking, and we wanted to include these factors in the models. After 20 years of diabetes duration the incidence of DN will drop (1, 4, 27), but a longer duration will increase the prevalence of DN in any diabetes population so duration of diabetes is clearly associated with DN, as is age, since with higher age often comes longer duration.

Age at diabetes onset has been investigated in relation to microvascular complications such as microalbuminuria and diabetic retinopathy. Puberty could play an important role in the development of diabetic complications due to changes in growth hormones and glycemic control (221). In children diagnosed with diabetes at or after puberty, the development of microalbuminuria seems to be constant over time, but in children with diabetes onset before puberty there is a period of latency followed by more rapid development of microalbuminuria after onset of puberty (222). An early diabetes onset (before 5 years of age) may delay the time to onset of retinopathy, and a recent study also showed that prepubertal onset of T1D prolonged the time to onset of end stage renal disease (223), meaning that the youngest diabetic patients are somewhat protected against developing microvascular complications during childhood (224, 225). Age, age at onset of diabetes and diabetes duration are intercorrelated, so all three does not need to be included. Age at diabetes onset was included in the logistic regressions in study IV and V, but in study II the age of the participants was significantly different in the case and control groups, while age at onset was not, therefore age was included in the logistic regression but not age at onset. It must be recognized also that the study design, choosing controls with a different duration interval (>20yrs) than for the cases, will somewhat affect the modelling of both age and age at onset.
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Whether male or female sex increases the risk of DN has been disputed. Non-diabetic renal diseases progress slower in females (226), but this is not established in diabetic renal disease (227, 228). In the present studies, male sex was associated with a doubled risk of DN. Since these are case-control studies, we can speculate that men without renal complications are less motivated to participate in studies than women without renal complications and thus bias the sample set, but this cannot explain the large risk increase found among men.

Heritability for hypertension and cardiovascular disease has been found to increase the risk of developing DN (45-48). An elevated blood pressure leads to higher strain on the kidneys through an increase in GFR, but damage to the kidneys per se will also cause an elevated blood pressure that can accelerate the progression of the disease. Thus it is difficult to know what comes first, hypertension or kidney damage, unless a cohort of patients is followed from diabetes onset. In a previous study from our group, young T1D patients were followed for more than 8 years and the results showed that elevated GFR predicted DN while the elevation of blood pressure came after onset of albumin excretion (8). The DN cases in all studies included in this thesis had significantly higher blood pressure than the control groups, despite most of the cases having antihypertensive treatment and the controls being untreated. If blood pressure values from before the onset of microalbuminuria had been accessible, we may have been able to determine if elevated blood pressure was a true risk factor for DN initiation in these patients, but unfortunately we only had access to the most recent values. The differences in treatment between cases and controls and the contribution of kidney damage to the elevation of blood pressure would argue against including these values in further analyses. On the other hand, the genes involved in blood pressure regulation could influence the DN risk via its effect on blood pressure. When systolic and diastolic blood pressure values were included in the logistic regression in study IV, together with age at onset, duration, smoking, HbA1c and sex, the NOS3 gene was associated independently with DN (paper IV).

In the dietary study, in addition to age, duration, smoking, HbA1c and sex, we also adjusted for a number of other variables. BMI and energy intake were adjusted for because they are correlated with intake of nutrients. Mean arterial pressure, MAP, was included since it could be a confounder
for microalbuminuria. It is approximated by using diastolic and systolic blood pressures (DP and SP), MAP=(2*DP+SP)/3. The region of origin was also included because it can be speculated that the diet differs between different parts of the country.

**Interactions**

In studies II, IV and V the combined effect of smoking and genetic polymorphisms were investigated. Statistical interaction, or effect modification, can be either on the multiplicative or the additive scale. Multiplicative interaction is when the combined effect of two variables is different from the product of the effects of the two, while additive interaction is when the combined effect of two variables differs from the sum of the effects of the two individual factors (229, 230). In a biological system the term interaction describes two (or more) factors that are interdependent in causing or preventing an effect (229). In public health or individual risk estimations it has been suggested that the additive scale is more accurate to use (231). Therefore the additive scale was used in the present studies. The method chosen for assessing interaction was the RERI, also called the interaction contrast ratio (229). The RERI is the excess risk due to interaction, relative to the risk without exposure. The risk without exposure to any of the factors is set to 1. The other risks, associated with either of the exposures or both, are calculated relative to that of no exposure. The excess risk due to interaction is the risk with two exposures minus the risks of the single exposures plus 1 (i.e. the risk when having no exposure) RERI=1+OR_{A+B+}−OR_{A+B−}−OR_{A+B+}. The results from the three studies suggested a possible synergistic effect, more than additive, of smoking and genes among the Swedish participants. When including the Finnish sample set in studies IV and V, these findings could not be repeated. A reason for the discrepant results may be different misclassification of smoking exposure. The percentage of smokers differed between the controls in the two sample sets, making the smoking exposure a very strong risk factor in the Finnish patients, perhaps overshadowing the modest contribution of the possible joint effect of gene and smoking. It is also a possibility that the observed synergism is due to statistical fluctuations in the smaller Swedish sample set.
RESULTS AND DISCUSSION

In a disease like DN, with complex genetic background, there can also be gene-gene interactions influencing the risk. However, no more than additive effect was detected when combining the SOD2 Val/Val- and NOS3 Glu/Glu-genotypes.

Correcting for multiple testing, to do or not to do?
Correction for multiple testing involves two types of considerations. The probability to find positive associations by chance, falsely reject the null hypothesis (type 1 error), and the risk to accept the null hypothesis falsely (type 2 error). When testing different genetic markers in the same population, type 1 error (false positive results) may be a problem due to multiple testing, and it may be relevant to decrease the level at which a probability is considered significant (decrease the risk of type 1 error). On the other hand this will at the same time increase the risk of type 2 error. There are ways to statistically correct for multiple testing and the Bonferroni correction is quite common. The Bonferroni correction adjusts the $\alpha$-value (the value of the probability distribution at which you wish to reject the null hypothesis, usually set to 0.05) thus reducing the risk of a type 1 error. With the correction this value is approximately $\alpha/n$, where $n$ is the number of tests performed (232). This type of correction leads to a very conservative decision, however, and when doing analyzes with a preformed hypothesis or when trying to repeat previous findings, it may bee too conservative (232, 233).

In study II, four different genes and seven different markers were tested. All markers had been investigated in relation to either DN or hypertension in previous studies, and we chose to partly correct for multiple testing by choosing a significance level of 0.01, instead of 0.05. When choosing $P=0.05$ as significance level, 1 in 20 statistical tests can show statistical significance by chance (false positive), while if $P=0.01$ is chosen, the chance for false positive is 1/100. With full Bonferroni correction, for all seven markers, $\alpha$ would have been set to 0.007.

In study III, one gene was tested using five different SNPs and a full Bonferroni correction would give $\alpha=0.01$. Some of the polymorphisms, however, were in linkage disequilibrium, which makes a full correction unnecessary, so no corrections were made. Studies IV and V included only two and one marker respectively, so there were no correction for multiple
testing made in these studies. Instead of using corrections we showed the exact P-values and risk estimates with confidence limits and gave our interpretation of the results, also pointing out that no corrections were made. This gives the reader a possibility to consider the significance of the results with regard to the hypothesis tested.

**Summary hypothesis of a complex pathogenesis**

Figure 11 shows a hypothetical summary of the effects of hyperglycaemia, smoking and dietary protein on the development of DN.

Excess glucose increases the activity of the polyol and hexosamine pathways, which increase the formation of oxygen radicals. Oxygen radicals can inhibit GAPDH and cause further increase of the intermediate glycolysis products. Hyperglycaemia also increases the formation of AGEs, activates PKC and a number of transcription factors, leading to increased expression of different growth factors and the anti-fibrinolytic PAI-1. In the end this results in accumulation of collagen and fibronec tin in the glomeruli and growth of the mesangium and basement membranes (fig. 11). *In vitro* models have shown that overexpression of MnSOD normalizes the effects of GAPDH inhibition (94, 101, 108). In study V we could find a small but significant association between risk of having DN and the Val/Val genotype, of the Val16Ala polymorphism in MnSOD.

Elevated glucose levels activates the intrarenal RAAS (234, 235), increases blood pressure, renal plasma flow and GFR, increasing the risk of developing DN. In study II we saw that the AGTR1 genotype can influence the risk of DN.

The eNOS enzyme, important for the regulation of blood flow in the glomeruli, is also affected by hyperglycaemia as well. Experiments have shown that eNOS expression is increased by insulin but reduced by PKC (102) and in study IV we associated a genetic polymorphism in this gene with increased risk of DN.

Smoking can be regarded as a known risk factor for DN, affecting both blood pressure and oxidative stress, and we could confirm the association with DN in our studies. There were also interesting findings of synergistic effects with the studied genes, suggesting that combinations of smoking and some genotypes can increase the risk. This could, however,
Figure 11: Hyperglycaemia increases the amount of intermediate glycolysis products, which activates the polyol and hexosamine pathways. Also PKC is activated and the formation of AGEs and ROS is increased. The RAAS is activated by excess glucose, among other things, and when incorrectly activated it may lead to hyperfiltration and hypertension. All these factors taken together will increase the risk of developing DN.
not be found in both the Swedish and Finnish sample sets and must be further investigated in other studies.

The immune system is involved in the development of T1D and it may also be part of the pathogenesis of diabetic complications. Inflammatory markers are elevated in diabetes, particularly in diabetes with complications (145, 236) and activation of the complement system has been associated with risk of DN (150). It is also known that inflammation is present in cardiovascular diseases, like atherosclerosis and stroke (237, 238), which can be part of the explanation for the increased cardiovascular mortality among patients with DN. In study III, two polymorphisms in the \textit{ICAM1} gene were associated with T1D but no significant association with DN was found.

Patients with kidney disease are recommended to lower their protein intake since a high protein intake increases the workload for the kidneys and increases GFR. Even if we did not find an association between high protein intake and microalbuminuria in study I, we cannot exclude that high protein intake can be a risk, especially for progression to later stages of nephropathy. On the other hand we found a significant protective effect of fish protein consumption, which may mirror an effect of fish fat consumption, or suggest that different protein sources have different renal effects.
Conclusions

- The AA-genotype of the A1166C polymorphism in the AGTR1 gene was associated with overt DN when adjusting for age, duration of diabetes, sex, ever smoking and HbA1c.

- Homozygosity for the Glu-allele of the NOS3 Glu298Asp polymorphism increased the risk to develop DN when adjusting for age at diabetes onset, duration of diabetes, sex, blood pressure, ever smoking and HbA1c.

- The Val/Val-genotype of the SOD2 V16A polymorphism was associated with increased risk of DN, when taking the other associated factors, age at diabetes onset, duration of diabetes, sex, ever smoking and HbA1c, into account.

- Two polymorphisms in the ICAM1 gene (E469K and a C/G exchange) were significantly associated with T1D, but not with DN.

- Smoking and the AGTR1 polymorphism had synergistic effects, further increasing the risk of overt DN. Synergistic effect with smoking was also indicated with the NOS3 and the SOD2 genes, in the Swedish patients but not in the Finnish.

- In young Swedish T1D patients a diet rich in fish was a significant protective factor and a normal protein intake did not increase the risk of having microalbuminuria, but we cannot exclude that a high-protein diet can have adverse effects.

In summary, the angiotensin receptor gene, the NOS3 and SOD2 genes were associated with increased risk of having DN, while the ICAM1 gene was associated with T1D but not with DN. Smoking was a risk factor for DN and possibly smoking can increase the risk associated with some genetic polymorphisms, but no unambiguous gene-smoking interactions were found. A normal protein intake did not increase the risk of having microalbuminuria, but diet containing lots of fish protein had a protective effect.
Final Comments

There is a need for concurrence of many factors for initiation and progression of DN and clearly both genetic-, environmental- and lifestyle-related factors are involved. Figure 12 illustrates hypothetical scenarios where three different combinations of risk factors can lead to the same result, development of DN.

![Figure 12: Illustration of hypothetical combinations of risk factors leading to DN. The genetic background can make patients susceptible to different environmental influences.](image)

Genetic background may influence the effects of poor metabolic control (58) and antihypertensive medication (239-241), but there are studies showing contradictory results (59, 242, 243). It is, however, difficult to assess the influence of one specific polymorphism since there are other polymorphisms that can contribute and also additional factors, like smoking, that affects the outcome. There is for example evidence that smoking interferes with antihypertensive treatment and causes increased risk of cardiovascular events in hypertensive patients under intensive treatment (244).

Another important aspect is timing, some factors can be important during initiation of DN and others are important for progression, or both. High protein intake, for example, can cause a faster progression of DN, and blood pressure is associated with progression of DN, but can also be involved in the initiation of disease. Each one of the factors mentioned in this thesis can have an impact on the outcome of the disease, but alone they are not enough, the combination is the key.
There is no simple explanation to why some but not all T1D patients develop DN. Increased risk can be associated with increased HbA1c, increased blood pressure and can be found among smokers, but not all patients with these risk factors will eventually develop DN, and patients without these factors are not fully protected from the complication. This thesis illustrates the importance of taking different variables into account, and hopefully the reported findings can help improve the understanding of the mechanisms behind the development of DN. Case-control studies detecting association of genetic polymorphisms with DN may seem far away from improving the everyday life of diabetic patients, but genetic predisposition to complications can be detected already at T1D onset and this will give a head start to the preventive medical consultation and treatment. Since changes in the glomeruli occur well before the onset of microalbuminuria (245), effective treatment is needed very early to prevent the initiation of DN. Perhaps also individualized treatment can be introduced, based on the genotype (246) and lifestyle of the patient. A good metabolic control is essential for all T1D patients and also to refrain from smoking, but it is still not clear which genetic factors are most important for DN development. Thus it is necessary to continue both the analyses of known candidate genes and the search for new candidates, to find combinations of genes that predispose for disease.
Populärvetenskaplig sammanfattning


Upp till 30 % av typ 1 diabetikerna drabbas av diabetisk njurskada (DN) vilket gör att njurarna slutar fungera och patienten behöver dialys och till sist njurtransplantation. Denna komplikation ökar också risken för hjärtkärlsjukdomar som hjärtinfarkt och stroke. Det finns många faktorer som påverkar risken att drabbas av DN. Dålig blodsockerkontroll är den viktigaste, men också rökning och förhöjt blodtryck kan öka risken. Alla med dålig sockerkontroll drabbas dock inte och även diabetiker med bra kontroll av sitt blodsocker kan drabbaras. Efter 15-20 år med typ 1 diabetes är risken som störst att drabbas och efter det minskar risken, vilket kan jämföras med andra komplikationer där risken ökar hela tiden. Detta tillsammans med resultat från familjestudier tyder på att vissa har ärftlig benägenhet att utveckla DN.

I denna avhandling var huvudsyftet att undersöka ett antal faktorer som kan öka risken för DN. Genom att jämföra en grupp typ 1 diabetiker som har DN med en grupp typ 1 diabetiker som inte har det, trots lång tid med diabetes, kan vi se om det finns skillnader i gener och livsstil som påverkar risken att få DN.

Studie I var inriktad på kostens betydelse. Många ämnen i kosten kan påverka njurarna, till exempel protein, fett och vitaminer (antioxidanter) men det var främst proteinets inverkan vi ville studera. Det är påfrestande för njurarna att göra sig av med nedbrytningsprodukter från proteiner och det är känt att om man äter mycket protein kan det öka risken för njursvikt. Så många som 1150 unga typ 1 diabetiker från hela Sverige fyllde i en enkät om sina matvanor under de senaste 12 månaderna. Alla testades sedan för protein i urinen, vilket är ett tecken på njurpåverkan. Bland deltagarna fanns 75 med förhöjt värde (≥15μg/min), de räknades som fall. Tre kontroller per
POPULÄRVETENSKAPLIG SAMMANFATTNING


I studie II undersöktes fem gener i renin-angiotensin-aldosteron systemet, ett system som är viktigt för blodtrycksregleringen och som kan agera lokalt i njurarna. I denna studie deltog typ 1 diabetiker med diabetisk njurskada i olika stadijer, från protein i urinen ($\geq 20 \mu g/min$) till njurtransplanterade patienter. Kontrollerna hade haft diabetes i minst 20 år, de var inte behandlade med blodtrycksmediciner och hade inget protein i urinen (<20 $\mu g/min$). Sju genetiska markörer jämfördes mellan fall och kontroller och vi fann att en markör i receptorn för angiotensin var associerad med tre gånger förhöjd risk för DN vid justering för de andra faktorerna; ålder, kön, blodsockerkontroll, rökning och år med diabetes. Vi fann också att den risk för DN som var associerad med den genetiska markören var ännu högre hos rökare.

I Studie III studerades genen som kodar för adhesionsmolekylen ICAM-1. ICAM-1 är viktig vid inflammation, då vita blodkroppar ska aktiveras och förflytta sig dit de behövs för att skydda mot t.ex. bakterier, men vid typ 1 diabetes ger sig blodkropparna på de egna insulinproducerande cellerna. Två polymorfier i denna gen ökade risken för typ 1 diabetes (1.6 och 2.5 gångers riskökning) men ingen av polymorfierna var associerad med DN.

I studie IV undersöktes genen $NOS3$, som kodar för ett enzym som tillverkar NO. NO är en kärlavslappnande molekyl som hjälper till att reglera blodtrycket. Om det finns NO slappnar blodkärlen av och blodtrycket sänks, men det kan samtidigt göra att blodflödet ökar, vilket kan öka risken för skada i njuren. I denna studie ingick både svenska och finska typ 1 diabetiker. En av de två studerade polymorfierna var vanligare hos
patienter med DN då även följande faktorer räknades med; ålder vid diabetesdebut, år med diabetes, blodtryck, kontroll av blodsocker, kön och rökning. Ungefär 1.5 gångers riskökning associerades med den genvarianten. I den svenska gruppen fanns en tendens till synergistisk effekt av gen och rökning, men i den finska gruppen hade rökning ingen modifierande effekt.


Sammanfattningsvis fann vi tre gener, angiotensin receptor genen, NOS3 och SOD2, som var associerade med DN medan ICAM1 kunde associeras med typ 1 diabetes men inte med DN. Rökning kunde också associeras med DN och tendenser till synergier hittades med genpolymorfier, men endast bland de svenska deltagarna. Bland unga diabetiker hade hög fiskkonsumtion en skyddande effekt mot protein i urinen. Det fanns inte någon ökad risk för de som i en normal diet åt mer protein, men vi kan inte utesluta att ett ännu högre intag av protein kan påverka njurarna negativt.

Det är viktigt att minska antalet diabetiker som drabbas av DN och för att göra det behövs tidig behandling med till exempel blodtryckssänkande mediciner. Utifrån studierna som ingår i denna avhandling kan vi se att många olika saker medverkar till risken att få njurkomplikation vid diabetes, både genetiska faktorer och livsstil kan ha betydelse. Förhoppningsvis kan genetiska studier öka förståelsen för de inblandade mekanismerna och i framtiden ge möjlighet att individanpassa förebyggande behandling.
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